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(54) Title: CANINE RESPIRATORY CORONAVIRUS (CRCV) SPIKE PROTEIN, POLYMERASE AND HEMAGGLU-  
TININ/ESTERASE

ctcagatgaa tttgaaatat gctatttagtg ctaagaatag agcccgcaact gttgctgggtg 60  
tttccataact tagtactatg actggcagaa tgtttcatca aaaatgtttg aaaagtatatg 120  
cagctacaacg tgggtgttcct gttgttatag gcaccactaa attttatggc ggctggggatg 180  
atatgttacg tcgccttatt aaagatgttg acaatectgt acctatgggt tgggattatc 240  
ctaagtgtga 250

(57) Abstract: A canine respiratory coronavirus (CRCV) that is present in the respiratory tract of dogs with canine infectious res-  
piratory disease and which has a low level of homology to the enteric canine coronavirus, but which has a high level of homology  
to all bovine coronavirus strains (eg Quebec and LY138) and human coronavirus strain OC43. The CRCV spike, polymerase and  
hemagglutinin/esterase cDNA and protein partial sequences are listed in Figures (1) to (4), (13) and (14).

**CANINE RESPIRATORY CORONAVIRUS (CRCV)  
SPIKE PROTEIN, POLYMERASE AND  
HEMAGGLUTININ/ESTERASE**

The present invention relates to biological material, and in particular to a canine respiratory coronavirus that is present in dogs having canine infectious respiratory disease.

- 5 Canine infectious respiratory disease (CIRD) is a highly contagious disease common in dogs housed in crowded conditions such as re-homing centres and boarding or training kennels. Many dogs suffer only from a mild cough and recover after a short time, however in some cases a severe bronchopneumonia can develop (Appel and Binn, 1987).
- 10 The pathogenesis of CIRD is considered to be multifactorial, involving several viruses and bacteria. The infectious agents considered to be the major causative pathogens of CIRD are canine parainfluenzavirus (CPiV) (Binn *et al.*, 1967), canine adenovirus type 2 (CAV-2) (Ditchfield *et al.*, 1962) and the bacterium *Bordetella bronchiseptica* (Bernis *et al.*, 1977, Keil
- 15 *et al.*, 1998). Also, canine herpesvirus, human reovirus and mycoplasma species have been isolated from dogs with symptoms of CIRD (Karpas *et al.*, 1968, Lou and Wenner 1963, Randolph *et al.*, 1993) Additional factors like stress may also be important.

CIRD is rarely fatal but it delays re-homing of dogs at rescue centres and it

20 causes disruption of schedules in training kennels as well as considerable treatment costs.

Vaccines are available against some of the infectious agents associated with this disease, namely *Bordetella bronchiseptica* as well as CPiV and CAV-2. However, despite the use of these vaccines, CIRD is still prevalent in

25 kennels world-wide, which is possibly due to the vaccines not providing protection against all the infectious agents involved in CIRD.

We have discovered a novel coronavirus, which we have called canine respiratory coronavirus (CRCV), in a large kennelled dog population with a history of endemic respiratory disease, and we have shown that this virus is associated with CIRD.

- 5 Some members of the family *coronaviridae* are known to cause respiratory disease in humans, cattle, swine and poultry (Mäkelä *et al.*, 1998, Pensaert *et al.*, 1986, Ignjatovic and Sapats 2000). For example, bovine respiratory coronavirus is associated with shipping fever in cattle which is a multifactorial respiratory disease (Storz *et al.*, 2000).
- 10 However, coronaviruses were not suspected to have a role in the pathogenesis of CIRD. Indeed, with only a single exception, canine coronaviruses have been reported to be enteric viruses and to cause acute diarrhoea mainly in young dogs (for example, Tennant *et al.*, 1993). In a large study of viruses involved in canine respiratory diseases, Binn *et al.*
- 15 (1979) reported the detection of a canine coronavirus in the lung of a single dog that was also infected with SV5 and canine adenovirus 2, two other viruses that are associated with canine respiratory disease.

There are 30-40 dog vaccines commercially available in the UK for use against a number of pathogens that can cause a range of diseases, such as

20 neurological, enteric, hepatic and respiratory diseases. Most of these vaccines contain microbial agents such as Distemper virus, Canine Adenovirus-2, Canine parvovirus, canine parainfluenza virus and *Leptospira canicola* and *L. icterohaemorrhagiae*. None of these vaccines contain canine coronaviruses.

- 25 The dog vaccines for use against canine respiratory diseases are marketed as vaccines for "kennel-cough" (see below). All of the vaccines contain

*Bordetella bronchisepticum*, which is a bacterium associated with "kennel cough".

Coyne M.J. & May S.W., (1995) in their article entitled "Considerations in using a canine coronavirus vaccine" (published as a Pfizer Technical Bulletin on the Internet at <http://www.pfizer.com/ah/vet/tref/trbull/ccv.html>), lists over 20 commercially available vaccines against either canine coronaviruses alone or against canine coronaviruses together with other organisms. Each of these vaccines is for canine enteric disease, and there is no suggestion that a canine coronavirus may be associated with respiratory disease.

US Patents Nos. 6,057,436 and 6,372,224, both to Miller *et al* and assigned to Pfizer, Inc., describe the spike gene of the enteric canine coronavirus and uses therefor, including its use as a vaccine against gastroenteritis. Neither of these two patents suggest that a canine coronavirus may be involved in CIRD.

Members of the family *coronaviridae* are enveloped viruses, 80-160nm in diameter, containing a linear positive-stranded RNA genome. The structural proteins of coronaviruses are the spike glycoprotein (S), the membrane glycoprotein (M) and the nucleocapsid protein (N). The hemagglutinin/esterase glycoprotein (HE) is found only on the surface of group II coronaviruses (e.g. bovine coronavirus and murine hepatitis virus) (Spaan *et al*, 1988). Further details of the structure of coronaviruses may be found in the chapter by Cavanagh *et al* entitled "Coronaviridae" p407-411, in "Virus Taxonomy, 6<sup>th</sup> Report of the International Committee on Taxonomy of Viruses", pub. Springer-Verlag Wein, New York, Eds. Murphy *et al*, which is incorporated herein by reference.

The canine respiratory coronavirus (CRCV) of the invention may be characterised as a coronavirus present in the respiratory tracts of dogs with infectious respiratory disease. To further characterise CRCV, we have determined the sequence of 250 nucleotide residues of the CRCV polymerase (pol) cDNA (Figure 1 and SEQ ID NO: 1) which corresponds to an 83 amino acid partial sequence of the pol protein (Figure 2 and SEQ ID NO: 2). We have also cloned and determined the sequence of the 4092 nucleotide residues of the CRCV spike (S) cDNA (Figure 3 and SEQ ID NO: 3), corresponding to 1363 amino acids (Figure 4 and SEQ ID NO: 4). We have also determined the sequence of 497 nucleotide residues of the CRCV hemagglutinin/esterase (HE) gene (Figure 13 and SEQ ID NO: 21), corresponding to 165 amino acids (Figure 14 and SEQ ID NO: 22). We have identified that CRCV has a surprisingly low homology to the enteric canine coronavirus (CCV) while it has an unexpectedly high level of homology to bovine coronavirus (strain LY138 or Quebec) and human coronavirus (strain OC43).

A culture of "Spike D-1 CRCV", which is XL1-Blue *E. coli* (Stratagene) containing a pT7Blue2 plasmid (Novagen) whose insert contains a portion of the CRCV spike cDNA, has been deposited under the Budapest Treaty at NCIMB Ltd under Accession number NCIMB 41146 on 25 July 2002. The depositor of NCIMB 41146 is the Royal Veterinary College, Royal College Street, London NW1 OTU, UK. The address of NCIMB Ltd is 23 St. Machar Drive, Aberdeen, Scotland, AB24 3RY, UK.

The phylogenetic relationship of CRCV to eleven known coronaviruses was determined based upon a comparison of the 250 nucleotide sequence from the CRCV pol gene and the corresponding regions of the other viruses (Figure 5). The bovine coronavirus (BCV), human coronavirus (HCV) strain OC43 and hemagglutinating encephalomyelitis virus (HEV) were

found to be most closely related to CRCV, while the enteric CCV was found to be only distantly related to CRCV.

Over the 250 sequenced residues of the pol cDNA, corresponding to 83 amino acids, CRCV has only 68.5% and 75.9% sequence identity at the nucleotide and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) pol gene (Genbank Accession No. AF124986), as shown in Figure 6 and 7.

Over the 4092 sequenced nucleotide residues of the CRCV S gene, corresponding to 1363 amino acids, CRCV has 45% and 21.2% sequence identity at the nucleotide (Figure 8) and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) S gene.

Enteric CCV is not a group II coronavirus and does not possess an HE gene, hence it is not possible to determine the extent of sequence identity between this gene in CRCV and in enteric CCV.

Except as described below, the percentage identity between two nucleotide or two amino acid sequences was determined using FASTA version 34 (Pearson WR. (1990) "Rapid and sensitive sequence comparison with FASTP and FASTA". *Methods Enzymol.*;183:63-98). FASTA settings were Gap open penalty -16 and Gap extension penalty -4.

The percentage identity between the CRCV and enteric CCV spike sequences was determined using GCG version 10 (Genetics Computer Group, (1991), Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, Wisconsin, USA 53711). The GCG parameters used were: Gap creation penalty 50, gap extension penalty 3 for DNA, and Gap creation penalty 8 and Gap extension penalty 2 for Protein.

Sequence alignments were performed using ClustalX (Thompson *et al.*, 1997).

By contrast, over the 250 sequenced residues of the pol cDNA, CRCV has 98.8% sequence identity with the equivalent region of the BCV strain Quebec pol gene (Genbank Accession No. AF220295), 98.4% sequence identity with the BCV strain LY138 pol gene (Genbank Accession No. AF124985) and 98.4% sequence identity with the HCV OC43 pol gene (Genbank Accession No. AF124989).

There was only a single amino acid difference between the CRCV pol protein over the 83 sequenced amino acids and the BCV, HCV and HEV pol proteins which is that CRCV has E (Glu) as opposed to D (Asp) at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29). Thus the CRCV pol protein is 99% identical to the BCV, HCV and HEV pol proteins over this region.

The one and three letter amino acid codes of the IUPAC-IUB Biochemical Nomenclature Commission are used herein.

Over the 497 sequenced nucleotide residues, corresponding to 165 amino acids, of the HE gene, CRCV has 98.994% and 98.2% sequence identity with the equivalent region of the BCV strain LY138 HE gene (Genbank Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 98.189% (nucleotide) and 98.2% (amino acid) sequence identity with human enteric coronavirus (HECV) HE gene (Genbank Accession No. L07747); 97.4% (nucleotide) and 95.2% (amino acid) sequence identity with the HCV OC43 HE gene (Genbank Accession No. M76373); and 92.0% (nucleotide) and 93.9% (amino acid) identity with HEV (Genbank Accession Nos. AF481863), as shown in Figures 15 and 16.

As shown in Figure 16 and Table 3, the three amino acids that are different between the CRCV HE protein and each of the BCV, HECV, HCV and HEV S proteins, within the 165 amino acids of the CRCV HE protein, are F (Phe) as opposed to L (Leu), N (Asn) as opposed to T (Thr), and L (Leu) as opposed to V (Val) at positions corresponding to position 235, 242 and 253, respectively, in the BCV, HECV, HCV OC43 and HEV HE genes (Figure 16). Thus F at position 235, N at position 242 and L at position 253 could be said to be CRCV HE protein-specific amino acids.

Over the 4092 sequenced nucleotide residues, corresponding to 1363 amino acids, of the CRCV S gene, CRCV has 97.3% and 96% identity with the equivalent region of BCV strain LY138 (Genbank Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 96.9% (nucleotide) and 95.2% (amino acid) identity with HCV strain OC43 (Genbank Accession No. Z32768), and 83.8% (nucleotide) and 80.4% (amino acid) identity with HEV (Genbank Accession Nos. AF481863 (cDNA) and AAM 77000 (protein)) as shown in Figures 9 and 10.

The amino acids that are different between the CRCV S protein and each of the BCV, HCV and HEV S proteins, within the 1363 amino acids of the CRCV S protein, are listed in Table 1 below. Thus the amino acids listed in Table 1 could be said to be CRCV S protein-specific amino acids. The amino acids are numbered from the initial M residue at the start of the CRCV protein, as shown in Figure 4.



**Table 1:** List of 39 amino acids specific to the CRCV S protein that are not present in the BCV, HCV and HEV S proteins.

<u>Position</u>	<u>Amino acid</u>
103	V
118	V
166	D
171	M
179	K
192	P
210	S
235	H
267	F
388	F
407	M
436	S
440	I
447	I
501	F
525	Y
528	N
540	L
582	K
608	G

<u>Position</u>	<u>Amino acid</u>
692	G
695	S
757	W
758	G
763	Q
769	T
786	P
792	H
818	R
827	P
828	V
887	F
933	D
977	F
1011	T
1018	S
1063	K
1256	L
1257	M

A first aspect of the invention provides a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein whose amino acid sequence is listed in Figure 4, and having at least one of V at position 103; V at position 118; D at position 166; M at position 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at position 758; Q at position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256; and M at position 1257. The amino acids are numbered from the initial M at the start of the CRCV S protein, as listed in Figure 4 (SEQ ID NO: 4).

It is appreciated that the partial nucleotide sequence of CRCV S can be readily determined by a person of ordinary skill in the art by sequencing the insert of the plasmid contained in *E. coli* strain D-1 CRCV, that has been deposited under the Budapest Treaty at NCIMB Ltd. under Accession number NCIMB 41146 on 25 July 2002. Furthermore, this DNA can be used as a hybridisation probe, or as the basis for the design of probes, in the isolation of CRCV nucleic acid in dogs.

For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), and comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 1.

By "protein" we also include the meaning glycoprotein. The amino acid sequence of a glycoprotein refers to the amino acid sequence of the polypeptide backbone of the glycoprotein, irrespective of the type, number, sequence and position of the sugars attached thereto.

- 5 Typically, the invention includes an isolated or recombinant protein, and not an unmodified CRCV protein present as a CRCV component.

The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), or at least 77%, or at least 78%, or at least 79%, or at least 80%,  
10 or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with the CRCV S protein, and  
15 comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85% or at least 90% or at least 95% amino acid sequence identity with the CRCV S protein (SEQ ID  
20 NO: 4), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or  
25 at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

Thus the invention includes a BCV, HCV or HEV S protein or fragment thereof, that has been modified at at least one position listed in Table 1 to resemble the CRCV S protein.

Preferably, the coronavirus S protein of the invention is a CRCV S protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4), or a variant thereof with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Thus the variant of the coronavirus S protein of the invention includes a protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4) but has between 1 and 40 amino acid differences from the sequence listed in Figure 4. Preferably, the variant has less than 40 amino acid differences from the sequence listed in Figure 4. More preferably the variant has less than 35, less than 30, or less than 25, or less than 20, or less than 15, or 10 or 9 or 8 or 7 or 6 or 5 or 4 or 3 or 2 amino acid differences, or a single amino acid difference, from the sequence listed in Figure 4.

The invention also includes a CRCV S protein fragment comprising a fragment of the sequence listed in Figure 4 (SEQ ID NO: 4) which comprises at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14, Genbank Accession No. AF058942), and comprising at least one of V at position 103; V at position 118; D at position 166; M at position 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; ; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at position 758; Q at position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256 and M at position 1257.

For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with BCV strain LY138 S protein, or at least 77%, or at least 78%, or at least 79%, or at least 80%, or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 S protein, and

having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

A second aspect of the invention provides a coronavirus pol protein, or fragment thereof, having at least 90% amino acid sequence identity with the BCV pol protein (SEQ ID NO: 5) and comprising the amino acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

The invention includes a coronavirus pol protein, or fragment thereof, having at least 91% amino acid sequence identity with BCV strain LY138 pol protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 pol protein, and having the amino

acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

Preferably, the coronavirus pol protein, or fragment thereof is a CRCV pol protein or fragment thereof that comprises or consists of the amino acid  
5 sequence listed in Figure 2.

Thus the invention includes a BCV, HCV or HEV pol protein or fragment thereof, that has been modified at the amino acid corresponding to position 4975 in the BCV genome, to resemble the CRCV pol protein.

The invention also includes a CRCV pol protein fragment comprising a  
10 fragment of the sequence listed in Figure 2 (SEQ ID NO: 2) and having the amino acid E at the position corresponding to position 4975 in the BCV genome.

A third aspect of the invention provides a coronavirus HE protein, or fragment thereof, having at least 90% amino acid sequence identity with the  
15 BCV LY138 HE protein (Genbank Accession No. AF058942), and having at least one of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention includes a coronavirus HE protein, or fragment thereof,  
20 having at least 91% amino acid sequence identity with BCV strain LY138 HE protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having at least one of F at position 235; N at position 242; and L at position 253. The  
25 amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention also includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having two of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention further includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having all three of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

Preferably, the coronavirus HE protein, or fragment thereof is a CRCV HE protein or fragment thereof that comprises or consists of the amino acid sequence listed in Figure 14 (SEQ ID NO: 22).

Thus the invention includes a BCV, HCV, HECV or HEV HE protein or fragment thereof, that has been modified at one or more of the amino acids corresponding to position 235, 242; and 253 to resemble the CRCV HE protein.

The invention also includes a CRCV HE protein fragment comprising a fragment of the sequence listed in Figure 14 (SEQ ID NO: 22) and having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in



which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

The coronavirus S, pol and HE proteins as defined above in the first, second and third aspects of the invention may be termed herein "CRCV" or  
5 "CRCV-like" proteins.

A "CRCV S protein" is an S protein or fragment thereof that has the native CRCV S amino acid sequence as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof which comprises at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

10 A "CRCV pol protein" is a pol protein or fragment thereof that has the native CRCV pol amino acid sequence as listed in Figure 2 (SEQ ID NO: 2), or a fragment thereof which comprises the amino acid E at the position corresponding to position 4975 in the BCV genome.

15 A "CRCV HE protein" is an HE protein or fragment thereof that has the native CRCV HE amino acid sequence as listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof which comprises one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the  
20 initial M at the start of the BCV LY138 HE protein.

A "CRCV-like S protein" is an S protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but has at least 75% sequence identity with the corresponding region of the CRCV or BCV strain LY138 S  
25 protein, and has at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

A "CRCV-like S protein" also includes an S protein that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but that comprises or consists of a variant of the sequence listed in Figure 4 with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

A "CRCV-like pol protein" is a pol protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV pol amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 pol protein, and which has an E at the position corresponding to position 4975 in the BCV genome.

A "CRCV-like HE protein" is an HE protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV HE amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 HE protein, and which has one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 10 amino acids in length. More preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400, or at

least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000, or at least 1,100, or at least 1,200 amino acids in length.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is less than about 1,300 amino acids in length. More preferably, the CRCV or  
5 CRCV-like protein, or fragment thereof, is less than about 1,200, or less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500, or less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

10 CRCV proteins may be isolated from CRCV, or may be made using protein chemistry techniques for example using partial proteolysis of isolated proteins (either exolytically or endolytically), or by *de novo* synthesis. Alternatively, the CRCV proteins, as well as CRCV-like proteins, may be made by recombinant DNA technology. Suitable techniques for cloning,  
15 manipulation, modification and expression of nucleic acids, and purification of expressed proteins, are well known in the art and are described for example in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3<sup>rd</sup> edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by  
20 reference.

Shorter fragments of CRCV and CRCV-like proteins, *ie* peptides, may be synthesised using standard techniques. Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* 46, 3433 and references therein. Temporary N-amino  
25 group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine

threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where  
5 glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethylacrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and  
10 acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexylcarbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All  
15 coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are  
20 ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a  
25 simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance  
30

liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

- 5 A fourth aspect of the invention provides a polynucleotide that encodes a CRCV or CRCV-like S, pol or HE protein according to the first, second and third aspects of the invention, or the complement thereof.

Preferably, the polynucleotide encodes a CRCV S protein according to the first aspect of the invention, or the complement thereof.

- 10 More preferably, the polynucleotide encoding the CRCV S protein comprises or consists of the sequence listed in Figure 3 (SEQ ID NO: 3).

It is appreciated that the sequence listed in Figure 3 (SEQ ID NO: 3) contains a Y at position 3531, which refers to either C or T. In both cases the corresponding amino acid is Ile. Thus the invention includes a  
15 polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having C at position 3531. The invention also includes a polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having T at position 3531.

- 20 The invention also includes a CRCV S polynucleotide comprising a fragment of the sequence listed in Figure 3 (SEQ ID NO: 3), that encodes a protein having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1, or the complement thereof.

Preferably, the polynucleotide encoding the pol protein comprises or  
25 consists of the sequence listed in Figure 1 (SEQ ID NO: 1), or the complement thereof.

The invention also includes a CRCV pol polynucleotide comprising a fragment of the sequence listed in Figure 1 (SEQ ID NO: 1) that encodes a protein having E at the position corresponding to position 4975 in the BCV genome, or the complement thereof.

- 5 Preferably, the polynucleotide encoding the HE protein comprises or consists of the sequence listed in Figure 13 (SEQ ID NO: 21), or the complement thereof.

The invention also includes a CRCV HE polynucleotide comprising a fragment of the sequence listed in Figure 13 (SEQ ID NO: 21) that encodes  
10 a protein having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

- 15 The polynucleotides as defined above are referred to herein as CRCV or CRCV-like polynucleotides of the invention.

A "CRCV-like polynucleotide" is a polynucleotide that does not have a base sequence identical to all or a fragment of the native CRCV cDNA sequence as listed in Figures 1, 3 and 13 (SEQ ID NOS: 1, 3 and 21), but  
20 that encodes a CRCV or CRCV-like S pol or HE protein as defined above, or the complement thereof.

The CRCV is a positive strand RNA virus. The polynucleotide of the invention may be DNA or RNA. The RNA may be positive or negative strand RNA. The DNA may be single or double stranded DNA.

- 25 Suitable techniques for cloning and sequencing a cDNA from a positive strand RNA virus such as CRCV are well known in the art and are

described for example in Sambrook *et al* 2001, incorporated herein by reference.

The CRCV or CRCV-like polynucleotides of the invention may be any suitable size. However, for certain purposes, such as probing or amplifying, it is preferred if the nucleic acid has fewer than 3,000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA oligonucleotides, suitable for use as hybridisation probes or as primers in a polymerase chain reaction, are particularly preferred.

Oligonucleotides that can specifically amplify, or hybridise to CRCV S, pol or HE polynucleotides, as opposed to BCV, HCV, HEV or enteric CCV S, pol or HE polynucleotides, are particularly preferred. Suitable oligonucleotides can be determined by a person of skill in the art by reference to the nucleotide sequence comparisons in Figures 6, 8, 9 and 15.

It is appreciated that the CRCV or CRCV-like oligonucleotides may, even under highly stringent conditions, hybridise to nucleic acid, whether RNA or DNA, from HCV, BCV, and HEV as well as from CRCV. However, it is preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from HCV, BCV or HEV. This can either be determined experimentally or by a comparison of the oligonucleotide sequence with the respective CRCV, HCV, BCV and HEV sequences, as is well known to one of skill in the art (Sambrook *et al* 2001).

It is also appreciated that the CRCV or CRCV-like oligonucleotides may hybridise to nucleic acid, whether RNA or DNA, from the enteric CCV as

well as from CRCV. However, it is preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from enteric CCV. This can either be determined experimentally or by a comparison of the oligonucleotide sequence with the respective sequences, as is well known to one of skill in the art (Sambrook *et al* 2001). Preferably, the oligonucleotides do not hybridise to nucleic acid from enteric CCV at all under stringent conditions (see below).

Conveniently, the CRCV or CRCV-like polynucleotides or oligonucleotides further comprise a detectable label.

By "detectable label" is included any convenient radioactive label such as  $^{32}\text{P}$ ,  $^{33}\text{P}$  or  $^{35}\text{S}$  which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless *o*-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed array and whether a nucleic acid hybridises to it can be determined by reference to the position of hybridisation in the fixed array.

Labelling with [ $^{32}\text{P}$ ]dCTP may be carried out using a Rediprime<sup>®</sup> random primer labelling kit supplied by Amersham.



Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* 239, 487-491) are preferred. Suitable PCR primers may have the following properties:

5 It is well known that the sequence at the 5' end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3' ends, as this feature may promote the formation of an artefactual product called "primer dimer". When the 3' ends of the two primers hybridise, they form a  
10 "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature  
15 calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple  $T_m$  calculations.

20 Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilised. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1µM  
25 range.

It will further be appreciated that if a control amplification reaction is to be carried out, for example using primers complementary to an ubiquitously expressed gene, that it may be beneficial for the products of the control and CRCV or CRCV-like products to be of different sizes, such that the two products may be distinguished by the detection means employed, for example by mobility on agarose gel electrophoresis. However, it may be desirable for the two products to be of similar size, for example both between 100 and 1000, or between 100 and 600 nucleotides long. This may aid simultaneous analysis of the products, for example by gel electrophoresis, and may also mean that the control and CRCV or CRCV-like amplification reactions may have similar performance characteristics, in terms, for example, of relative rates of accumulation of product at different stages during the reaction.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton (1991) *Nature* 350, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* 20, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long,

more preferably between 15 and 30 nucleotides long. It may be longer than the amplified DNA or include one or both of the primers, but in this case, the hybridisation conditions should be such that the probe should not hybridise to the primers alone, but only to an amplified product that also  
5 contains interprimer sequence that is capable of hybridising to the probe.

The probe may be labelled with a radionuclide such as  $^{32}\text{P}$ ,  $^{33}\text{P}$  and  $^{35}\text{S}$  using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991).  
10 "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* 195, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* 15, 152-157.

15 PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative  
20 measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

The invention also includes a vector comprising the CRCV or CRCV-like polynucleotide of the fourth aspect of the invention.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and  
25 pBR329 available from Biorad Laboratories (Richmond, CA, USA); pTrc99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from

Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

5 A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible  
10 promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA). Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating  
15 plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Generally, the CRCV or CRCV-like polynucleotide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and  
20 correct reading frame for expression. It may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host prior to insertion into the vector, although such controls are generally available in the expression vector. Thus, the polynucleotide of the invention insert may be operatively linked to an  
25 appropriate promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to the skilled artisan. The expression constructs desirably also

contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation (Hastings *et al*, International Patent No. WO 98/16643).

Methods well known to those skilled in the art can be used to construct expression vectors containing the coding sequence and, for example  
5 appropriate transcriptional or translational controls. One such method involves ligation via homopolymer tails. Homopolymer polydA (or polydC) tails are added to exposed 3' OH groups on the DNA fragment to be cloned by terminal deoxynucleotidyl transferases. The fragment is then capable of  
10 annealing to the polydT (or polydG) tails added to the ends of a linearised plasmid vector. Gaps left following annealing can be filled by DNA polymerase and the free ends joined by DNA ligase.

Another method involves ligation via cohesive ends. Compatible cohesive ends can be generated on the DNA fragment and vector by the action of  
15 suitable restriction enzymes. These ends will rapidly anneal through complementary base pairing and remaining nicks can be closed by the action of DNA ligase.

A further method uses synthetic molecules called linkers and adaptors. DNA fragments with blunt ends are generated by bacteriophage T4 DNA  
20 polymerase or *E.coli* DNA polymerase I which remove protruding 3' termini and fill in recessed 3' ends. Synthetic linkers, pieces of blunt-ended double-stranded DNA which contain recognition sequences for defined restriction enzymes, can be ligated to blunt-ended DNA fragments by T4 DNA ligase. They are subsequently digested with appropriate restriction enzymes to create  
25 cohesive ends and ligated to an expression vector with compatible termini. Adaptors are also chemically synthesised DNA fragments which contain one blunt end used for ligation but which also possess one preformed cohesive end.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the polynucleotide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The invention also includes a host cell transformed with the vector comprising the CRCV or CRCV-like polynucleotide. The host cell can be either prokaryotic or eukaryotic. If the CRCV or CRCV-like polynucleotide, in the vector, is to be expressed as a glycoprotein, the host cell is a eukaryotic host cell, and preferably a mammalian host cell.

Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1

cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a vector is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (2001) *Molecular Cloning, A Laboratory Manual*, 3<sup>rd</sup> Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5x PEB using 6250V per cm at 25 $\mu$ FD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194, 182.

Physical methods may be used for introducing DNA into animal and plant cells. For example, microinjection uses a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed. Another

example involves bombardment of the cells with high-velocity microprojectiles, usually particles of gold or tungsten that have been coated with DNA.

5 Successfully transformed cells, ie cells that contain a CRCV or CRCV-like DNA construct, can be identified by well known techniques. For example, one selection technique involves incorporating into the expression vector a DNA sequence (marker) that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin  
10 resistance genes for culturing in *E.coli* and other bacteria. Alternatively, the gene for the selectable trait can be on another vector, which is used to co-transform the desired host cell.

The marker gene can be used to identify transformants but it is desirable to determine which of the cells contain recombinant DNA molecules and which  
15 contain self-ligated vector molecules. This can be achieved by using a cloning vector where insertion of a DNA fragment destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because of loss of function of that gene.

Another method of identifying successfully transformed cells involves  
20 growing the cells resulting from the introduction of an expression construct of the present invention to produce the CRCV or CRCV-like S, pol or HE protein. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208.  
25 Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.



In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression  
5 vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal  
10 (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

Host cells that have been transformed by the recombinant CRCV or CRCV-like polynucleotide, typically in a vector as described above, are then cultured for a sufficient time and under appropriate conditions known to those skilled  
15 in the art in view of the teachings disclosed herein to permit the expression of the CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide, which can then be recovered.

The CRCV or CRCV-like protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium  
20 sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

25 For example, for expression in a baculovirus system, recombinant DNA encoding the CRCV spike gene may be cloned into a suitable transfer vector such as pMelBac (Invitrogen). Co-transfection with baculovirus DNA (eg

Bac-N-Blue/Invitrogen) results in a recombinant baculovirus encoding the spike gene. Infection of a suitable insect cell line (e.g. Sf9, Sf21, High Five/Invitrogen) at an appropriate multiplicity of infection leads to expression of the recombinant spike protein. Protein expression is confirmed by western blotting or ELISA using appropriate reagents (e.g. convalescent canine serum or other virus specific antiserum).

The invention thus includes a method of obtaining a CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide of the present invention. The method comprises culturing the host cell comprising the CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell, and purifying the protein. The invention further includes the protein obtainable by this method.

The invention thus also includes a method of obtaining a glycosylated CRCV or CRCV-like protein, typically an S protein, encoded by the CRCV or CRCV-like polynucleotide of the present invention. The method comprises culturing a eukaryotic, or more preferably mammalian, host cell comprising the CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell; and purifying the glycosylated protein. The invention further includes the glycosylated protein obtainable by this method.

In a fifth aspect, the invention provides a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention in an animal, and preparing an antibody from the animal or from an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention.

Methods and techniques for producing a monoclonal antibody are well known to a person of skill in the art, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and Applications*", J G R Hurrell (CRC Press, 1982), incorporated herein by  
5 reference.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV S protein than for the BCV S protein, and preferably also whether the antibody has a greater affinity for  
10 the CRCV S protein than for the HCV and HEV S proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the  
15 CRCV S protein than for the BCV S protein. Preferably, the antibody also has a greater affinity for the CRCV S protein than for the HCV and HEV S proteins.

The invention also includes a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like HE  
20 protein of the invention as described above in the third aspect of the invention in an animal, and preparing an antibody from the animal or from an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like HE protein of the invention as described above in the  
25 third aspect of the invention.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV HE protein than for the

BCV HE protein, and preferably also whether the antibody has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

- 5 The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the CRCV HE protein than for the BCV HE protein. Preferably, the antibody also has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins.
- 10 Preferably, the antibody is a monoclonal antibody. However, the invention includes a monospecific anti-CRCV antibody. The antibody may be an antibody fragment, as described below.

The monoclonal or monospecific antibody may be a chimaeric antibody, as discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799).  
15 The monoclonal or monospecific antibody may also be a "caninised" antibody, for example by inserting the CDR regions of mouse antibodies into the framework of canine antibodies.

The invention also includes anti-CRCV antibody fragments. The variable heavy ( $V_H$ ) and variable light ( $V_L$ ) domains of antibodies are involved in  
20 antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies, in which variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984)  
25 *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains.

- 5 These molecules include Fab-like molecules (Better *et al* (1988) *Science* 240, 1041); Fv molecules (Skerra *et al* (1988) *Science* 240, 1038); single-chain Fv (ScFv) molecules where the  $V_H$  and  $V_L$  partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* 242, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 5879) and single domain antibodies (dAbs)  
10 comprising isolated V domains (Ward *et al* (1989) *Nature* 341, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* 349, 293-299.

By "ScFv molecules" we mean molecules wherein the  $V_H$  and  $V_L$  partner  
15 domains are linked via a flexible oligopeptide.

The advantages of antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed.  
20 Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the fragments.

Whole antibodies, and  $F(ab')_2$  fragments are "bivalent". By "bivalent" we mean that the antibodies and  $F(ab')_2$  fragments have two antigen combining  
25 sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

In a sixth aspect, the invention provides a method of determining whether a dog has been exposed to CRCV. The method comprises obtaining a suitable sample from the dog, and identifying CRCV or an anti-CRCV antibody in the sample. The method may be used as an aid in the diagnosis  
5 of whether a dog has CIRDC.

The invention includes a method of detecting, in a sample obtained from a dog, past exposure of the dog to CRCV, the method comprising obtaining a suitable sample from the dog, and identifying anti-CRCV antibodies in the sample.

10 In one preferred embodiment, the suitable sample can be any antibody containing sample such as serum, saliva, tracheal wash or bronchiolar lavage.

Preferably, the anti-CRCV antibody can be detected using a BCV, HCV, HEV or CRCV antigen, more preferably, using a BCV or CRCV antigen.

15 More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein (Figure 4 and SEQ ID NO: 4); an S protein whose amino acid sequence is at least 75% identical with the amino acid  
20 sequence of the BCV S protein (Genbank Accession No. AF058942); HCV S protein (Genbank Accession No. L14643); to a coronavirus having an S protein at least 75% identical with BCV S protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus having an S protein at least 75% identical with the CRCV S protein, or a fragment thereof.

25 More preferably, identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino

acid sequence of the BCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the BCV S protein (Genbank  
5 Accession No. AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV S protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an S  
10 protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the CRCV S protein (Figure  
15 4 and SEQ ID NO: 4) or a fragment thereof.

Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like S protein as defined in the first aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample  
20 comprises identifying an antibody that selectively binds to the CRCV S protein as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof.

Similarly, identifying an anti-CRCV antibody in the sample may comprise identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence  
25 of the CRCV HE protein (Figure 14 and SEQ ID NO: 22); to an HE protein whose amino acid sequence is at least 90% identical with the amino acid

sequence of the BCV HE protein (Genbank Accession No. AF058942) or the HECV HE protein (Genbank Accession No. L07747); to a coronavirus having an S protein at least 90% identical with BCV HE protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus  
5 having an HE protein at least 90% identical with the CRCV HE protein, or a fragment thereof.

More preferably, identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the amino acid sequence of the BCV HE protein, comprises identifying an antibody  
10 that selectively binds to an HE protein whose amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the amino acid sequence of the BCV HE protein (Genbank Accession No.  
15 AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV HE protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an  
20 HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence of the CRCV HE protein, comprises identifying an antibody that selectively binds to an HE protein whose partial amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least  
25 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the partial amino acid sequence of the CRCV HE protein (Figure 13) or a fragment thereof.



Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like HE protein as defined in the third aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample  
5 comprises identifying an antibody that selectively binds to the CRCV HE protein whose partial amino acid sequence is listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof.

The invention includes a method of detecting CRCV in a sample obtained from a dog, the method comprising obtaining a suitable sample from the  
10 dog, and identifying CRCV in the sample.

It is appreciated that there may be some naturally occurring sequence variation between different isolates of CRCV. The invention thus includes identifying CRCV isolates whose S, pol and HE genes and proteins have some sequence variation from the sequences provided in Figures 1 to 4 and  
15 13 and 14. It is appreciated, however, that the same methods will be used to detect the variant isolates of CRCV, as well as the isolate characterised by the sequences listed in Figures 1 to 4 and 13 and 14.

In a preferred embodiment, the suitable sample can be a lung wash, tracheal wash, tonsillar swab or a biopsy or post-mortem sample from the respiratory  
20 tract of the dog.

Preferably, in this embodiment, identifying CRCV comprises identifying a nucleic acid component of CRCV.

Typically, this will be performed by extracting RNA from the sample, and obtaining cDNA therefrom, for example as is described in Example 1.  
25 Thereafter, a CRCV nucleic acid component is identified in the cDNA, for example using techniques involving high stringency hybridisation, specific

amplification, and nucleotide sequencing, as are well known to a person of skill in the art (Sambrook *et al* (2001) *supra*).

Preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the BCV genome, such as the LY138 strain genome (Genbank Accession No. AF058942) or a portion thereof.

Further preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the CRCV S, pol or HE polynucleotides (Figures 1, 3 and 13) or a portion thereof.

By "hybridising at high stringency" is meant that the polynucleotide and the nucleic acid to which it hybridises have sufficient nucleotide sequence similarity that they can hybridise under highly stringent conditions. As is well known in the art, the stringency of nucleic acid hybridisation depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridising sequences and on factors such as temperature, ionic strength and CG or AT content of the sequence.

Nucleic acids which can hybridise at high stringency to the CRCV cDNA molecule include nucleic acids which have >90% sequence identity, preferably those with >95% or >96% or >97% or >98, more preferably those with >99% sequence identity, over at least a portion of the CRCV cDNA.

Typical highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in Sambrook *et al* 2001 (*supra*), incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe nucleic acid is  $\geq 500$  bases is:

6 x SSC (saline sodium citrate)

0.5% sodium dodecyl sulphate (SDS)

100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the  
5 nucleic acid immobilised, may be washed at 68°C in 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H<sub>2</sub>O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H<sub>2</sub>O. Dispense into aliquots. Sterilise by autoclaving.

10 An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M sodium phosphate (pH 6.8)

15 1 mM EDTA (pH 7.6)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

0.1% non-fat dried milk

The optimal temperature for hybridisation is usually chosen to be 5°C  
20 below the T<sub>i</sub> for the given chain length. T<sub>i</sub> is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* 16, 4637 discusses the determination of T<sub>i</sub>s. The recommended hybridization temperature for 17-mers in 3M

TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

Preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a portion of the BCV genome (Genbank Accession No. AF058942).

More preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a portion of the CRCV S polynucleotide (Figure 3), or having at least 90%, or at least 95% identity with a portion of the CRCV pol polynucleotide (Figure 1), or having at least 90%, or at least 95% identity with a portion of the CRCV HE polynucleotide (Figure 13).

More preferably, identifying CRCV comprises identifying a CRCV polynucleotide as defined above with respect to the fourth aspect of the invention.

Most preferably, identifying CRCV comprises identifying a CRCV polynucleotide comprising or consisting of a sequence listed in Figure 1 or Figure 3 or Figure 13, or a fragment thereof.

In another preferred embodiment, identifying CRCV comprises identifying a protein component of CRCV.

Preferably, identifying a protein component of CRCV comprises identifying a CRCV protein as defined above in the first or second or third aspects of the invention.

Most preferably, identifying a protein component of CRCV comprises identifying a CRCV protein comprising or consisting of the amino acid sequence listed in Figure 2 or Figure 4 or Figure 14, or a fragment thereof.

Assaying a protein component of CRCV in a biological sample can occur using any art-known method. Preferred for assaying CRCV protein levels in a biological sample are antibody-based techniques.

Preferably, identifying a protein component of CRCV comprises using an antibody reactive with CRCV.

More preferably, the antibody reactive with CRCV is an anti-BCV antibody, an anti-HCV antibody, an anti-HEV antibody, or an anti-CRCV antibody obtainable or obtained by the methods of the fifth aspect of the invention.

For example, CRCV protein expression can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilise fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of CRCV protein for Western-blot or dot/slot assay (Jalkanen, M., *et al*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al*, *J. Cell. Biol.* 105:3087-3096 (1987)). In this technique, which is based on the use of cationic solid phases, quantitation of CRCV protein can be accomplished using isolated CRCV protein as a standard. This technique can also be applied to body fluid samples.

Other antibody-based methods useful for detecting CRCV protein expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a CRCV reactive monoclonal antibody can be used both as an immunoadsorbent and as an enzyme-labeled probe to detect and quantify

the CRCV protein. The amount of CRCV protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA for detecting a tumour antigen is described in Iacobelli *et al*, *Breast Cancer Research and Treatment* 11: 19-30 (1988). In another ELISA assay, two distinct specific  
5 monoclonal antibodies can be used to detect CRCV protein in a body fluid. In this assay, one of the antibodies is used as the immunoadsorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or  
10 "two-step" assay. The "one-step" assay involves contacting CRCV protein with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to  
15 immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with  
20 substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides  
enzymes, other suitable labels include radioisotopes, such as iodine ( $^{125}\text{I}$ ,  
25  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99\text{m}}\text{Tc}$ ), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In a seventh aspect, the invention provides an immunosorbent assay for detecting anti-CRCV S or HE antibodies. The assay comprises a solid phase coated with a CRCV or CRCV-like S or HE protein, or coated with both CRCV or CRCV-like S and HE proteins as defined in the first and third aspects of the invention, or obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid phase will bind to the protein; and a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

10 It is appreciated that an antigenic fragment of the CRCV or CRCV-like S protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV S antibody, and which comprises at least one of the amino acids specific for CRCV S protein as listed in Table 1.

15 It is also appreciated that an antigenic fragment of the CRCV or CRCV-like HE protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV HE antibody, and which comprises at least one of the three amino acids specific for CRCV HE protein as defined above.

Preferably, the CRCV or CRCV-like S or HE protein, or antigenic fragment thereof, that coats the solid phase is at least 10 amino acids in length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400 amino acids in length. The CRCV or CRCV-like S protein may be at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000 amino acids in length.

25 Preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, that coats the solid phase is less than about 1200 amino acids in

length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500 amino acids in length. The CRCV or  
5 CRCV-like S or HE protein may be less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

Preferably, the solid phase is a microtitre well.

Further preferably, the conjugate comprises anti-dog antibody.

- 10 Preferably, the conjugate comprises an enzyme, for example horseradish peroxidase. Further preferably, the immunosorbent assay also comprises a substrate for the enzyme.

Further details of suitable immunosorbent assays and ELISAs are provided above.

- 15 The invention includes a kit of parts which include the components of the immunosorbent assay. The kit of parts may thus include a solid phase such as a microtitre plate, CRCV or CRCV-like S or HE protein or both for coating the solid phase, a detectable label conjugate, such as an anti-dog antibody, which will bind to anti-CRCV antibodies bound to the solid  
20 phase. If the detectable label conjugate is an enzyme, the kit of parts may also include a substrate for the enzyme. The kit may also include a positive control sample that contains an anti-CRCV S or HE protein antibody, such as those described with reference to the fifth aspect of the invention, and a negative control sample.

- 25 The invention thus includes a solid substrate with a CRCV or CRCV-like S or HE protein as defined in the first and third aspects of the invention, or



obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV S or HE antibodies or both from a liquid sample, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid substrate will bind to the S or  
5 HE protein.

Typically, protein is coated on microtitre plates overnight at 4°C to 37°C, depending on the stability of the antigen. Unbound protein is washed off with a wash buffer such as phosphate buffered saline or Tris buffered saline. Serum or other samples are incubated on the plate, typically at 37°C for  
10 between 1 and several hours. Unbound material is washed off, the plates are incubated with enzyme-labelled (e.g. horseradish peroxidase) antibody, such as anti-canine IgG or IgM for serum samples, or anti-canine IgA for lung washes, for 1 to several hours at 37°C. Unbound antibody is washed off and plates are incubated with a substrate such as OPD for about 10 min,  
15 and the optical density measured in a photometer.

Preferably, the solid substrate is a microtitre well.

In an eighth aspect, the invention provides a vaccine composition for vaccinating dogs comprising (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus  
20 having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV  
25 protein or an immunogenic fragment thereof, or (vi) a coronavirus protein having at least 75% amino acid identity with a BCV protein or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof.

Preferably, the vaccine is packaged and presented for use in dogs.

When the vaccine comprises a coronavirus protein, or an immunogenic fragment thereof, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding  
5 portion of a BCV or CRCV protein.

Preferably, the coronavirus protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

Typical protein modifications include amino acid substitutions to improve the antigenicity of the vaccine. BCV, HCV and HEV proteins may be  
10 modified to be more like a CRCV protein. For example, the spike protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of differences shown in the comparison in Figure 10, or listed in Table 1. Additionally or alternatively, the HE protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of the three CRCV-  
15 specific residues as defined above.

Proteins in which one or more of the amino acid residues are chemically modified, may be used providing that the function of the protein, namely the production of specific antibodies *in vivo*, remains substantially unchanged. It is appreciated that synthesised proteins may be suitably modified before or  
20 after their synthesised. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism.

25 The protein may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently

antigenic themselves to obviate the use of a carrier. It may be advantageous for the protein to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the protein is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the protein of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys (SEQ ID NO: 52), beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different proteins of the invention may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present).

If the protein is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express it as a fusion product

with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

It is appreciated that the coronavirus component of the vaccine may be linked to other antigens to provide a dual effect.

- 5 Preferably, the coronavirus protein in the vaccine composition is an S protein. More preferably, the S protein is a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, an HEV S protein, or an immunogenic fragment thereof.
- 10 Most preferably, the vaccine composition contains a CRCV S protein that comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least at least 98%, or at least 99% amino acid sequence identity with the sequence listed in
- 15 Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

- Additionally or alternatively, the vaccine composition may comprise
- 20 coronavirus proteins such as a hemagglutinin-esterase protein (HE) or an integral membrane protein (M), or the small membrane protein (E) (Lai MMC & Cavanagh D, (1997) "The molecular biology of coronaviruses" *Adv. Vir. Res.*, 48: 1-100).

- In one embodiment, the HE, E or M proteins are BCV, HCV or HEV
- 25 proteins. In another embodiment, the HE, E or M proteins are CRCV proteins.

Preferably, the HE protein is a CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, or an immunogenic fragment thereof.

More preferably, the vaccine composition contains a CRCV HE protein that  
5 comprises or consists of the partial amino acid sequence listed in Figure 14, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 14. Preferably, the variant has at least at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 14. More preferably the variant has at least 99.1%, or at least  
10 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 14.

When the vaccine comprises a coronavirus, preferably the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or  
15 at least 95% amino acid identity with the BCV S protein. More preferably, the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the CRCV S protein.

Additionally or alternatively, when the vaccine comprises a coronavirus, preferably the coronavirus comprises an HE protein with at least 90% or at  
20 least 95% amino acid identity with the BCV HE protein. More preferably, the coronavirus comprises an HE protein with at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid identity with the CRCV HE protein.

In another preferred embodiment, the vaccine composition comprises a  
25 virus selected from BCV, HCV, HEV and CRCV, or a modification thereof.

It is appreciated that dog vaccines effective against a canine virus may be derived from a non-canine virus. For example US Patent No. 5,750,112 to Gill, and assigned to Solvay Animal Health Inc, discloses a vaccine against enteric canine coronavirus containing inactivated feline enteric coronavirus.

5 The disclosure of US 5,750,112 is incorporated herein by reference.

In one preferred embodiment, the virus is an inactivated virus. Methods for inactivating viruses for use in vaccines are well known in the art. Suitable methods include chemical methods, such as the use of beta proprio-lactone (BPL). Suitable inactivated bovine coronavirus vaccines may include  
10 inactivated BCV which is a component of bovine vaccines such as "Rotovec Corona" from Schering-Plough (<http://www.ukvet.co.uk/rotovec/scour.htm>); "Lactovac" by Hoechst Roussel Vet Ltd, (Veterinary Formulary 5th Edition of the Veterinary Data Sheet Compendium); "First Defense" by Immuncell Corp, USA; "Scour  
15 Bos 4" by Grand Laboraotries and "Scour Guard 3K" by Pfizer.

In an alternative embodiment, the virus is an attenuated virus. Methods for attenuating viruses for use in vaccines are well known in the art.

Preferably, the vaccine composition also comprises a pharmaceutically acceptable adjuvant.

20 Preferably, when the vaccine comprises a nucleic acid, the nucleic acid encoding the coronaviral protein or immunogenic fraction thereof, for use as a vaccine is a CRCV or CRCV-like S polynucleotide, or a CRCV or CRCV-like HE polynucleotide or both a CRCV or CRCV-like S and HE polynucleotide. More preferably, the nucleic acid comprises or consists of  
25 the nucleotide sequence listed in Figure 3 or Figure 13, or fractions thereof.

For vaccine use, the CRCV or CRCV-like S or HE nucleic acid can be delivered in various replicating (e.g. recombinant adenovirus vaccine) or non-replicating (DNA vaccine) vectors.

In a preferred embodiment, the vaccine may contain recombinant CRCV or  
5 CRCV-like S protein, as well as other immunogenic coronavirus proteins such as the HE protein.

As discussed above, several viral and bacterial agents are known to be associated with respiratory disease in dogs, including canine parainfluenza virus (CPIV), canine adenovirus type 2 (CAV-2), canine herpesvirus  
10 (CHV), and *Bordetella bronchiseptica* (*B. bronchiseptica*).

In another preferred embodiment, the vaccine may contain recombinant CRCV or CRCV-like S or HE protein, as well as other pathogenic organisms involved in respiratory disease of dogs such as canine parainfluenzavirus, canine adenovirus type 2, the bacterium *Bordetella*  
15 *bronchiseptica*, canine herpesvirus, human reovirus and mycoplasma species, or immunogenic proteins therefrom. Thus the vaccine may contain an agent capable of raising an immune response, such as the production of antibodies against CRCV, as well as against other pathogenic organisms involved in respiratory disease of dogs such as CPIV, CAV-2, *B.*  
20 *bronchiseptica* and CHV.

In an embodiment, as well as containing an agent capable of stimulating the production of antibodies against CRCV, such as a CRCV or CRCV-like S or HE protein, the vaccine composition further comprises any one or more of:

- 25 (a) an agent capable of raising an immune response in a dog against CPIV;

(b) an agent capable of raising an immune response in a dog against CAV-2;

(c) an agent capable of raising an immune response in a dog against CHV; and

5 (d) an agent capable of raising an immune response in a dog against *B. bronchiseptica*.

Thus the vaccine composition can optionally also comprise any two, or any three or all four of these additional agents (a), (b), (c) and (d).

Typically, an agent capable of raising an immune response in a dog against  
10 CPIV comprises inactivated or attenuated CPIV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Typically, an agent capable of raising an immune response in a dog against CAV-2 comprises inactivated or attenuated CAV-2, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

15 Canine adenovirus type 1 causes infectious hepatitis; canine adenovirus type 2 causes respiratory disease. It has been shown that CAV-1 provides cross-protection against CAV-2 and vice versa. The agent that raises an immune response in a dog against CAV-2 may therefore contain either CAV-1 or CAV-2, or an immunogenic fragment thereof. The vaccines  
20 listed below contain CAV-2 except for EURICAN DHPPI, which does not specify the virus type used.

Suitable agents that raise an immune response in a dog against CPIV and CAV-2 are known to a person of skill in the art. For example, the following dog vaccines are licensed in the UK.



KAVAK DA<sub>2</sub>PI69 by Fort Dodge Animal Health is a live freeze dried vaccine containing attenuated strains of canine distemper virus, canine adenovirus type 2, canine parainfluenza type 2 and canine parvovirus grown in tissue culture.

- 5 KAVAK Parainfluenza by Fort Dodge Animal Health contains live freeze-dried vaccine derived from an attenuated strain of canine parainfluenza virus type 2 cultivated on an established homologous cell-line.

- NOBIVAC DHPPi by Intervet UK Limited is a live attenuated freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus type 2,  
10 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

- NOBIVAC KC by Intervet UK Limited is a modified live freeze-dried vaccine containing *Bordetella bronchiseptica* strain B-C2 and canine parainfluenza virus strain Cornell (this is an intranasal vaccine).  
15 Management authorisation number Vm 06376/4026.

EURICAN DHPPi by Merial Animal Health Ltd. is a combined live freeze-dried vaccine against canine distemper, infectious canine hepatitis, canine parvovirus and canine parainfluenza virus type 2.

- VANGUARD 7 by Pfizer Ltd. contains live attenuated canine distemper  
20 virus (Snyder Hill strain), adenovirus (CAV-2 Manhattan strain), parainfluenza virus (NL-CPI-5 strain), canine parvovirus (NL-35-D) propagated in an established cell line, and an inactivated culture of *Leptospira canicola* and *Leptospira icterohaemorrhagiae*.

- QUANTUM DOG 7 by Schering-Plough Animal Health contains canine  
25 distemper, adenovirus type 2, parvovirus, parainfluenza virus type 2 vaccine

(living) and inactivated *Leptospira canicola* and *Leptospira icterohaemorrhagiae* vaccine.

CANIGEN DHPPi by Virbac Ltd. is a live attenuated, freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus (CAV2),  
5 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

CANIGEN Ppi by Virbac Ltd. is a live attenuated, freeze-dried virus vaccine containing canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

- 10 Typically, an agent capable of raising an immune response in a dog against CHV comprises inactivated or attenuated CHV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Suitable agents that raise an immune response in a dog against CHV are known to a person of skill in the art. For example, EURICAN Herpes 205  
15 by Merial is a purified sub-unit vaccine against canine herpesvirus which is indicated for the active immunisation of pregnant bitches to prevent mortality, clinical signs and lesions in puppies resulting from canine herpesvirus infections acquired in the first days of life. It is not licensed for the vaccination of adult dogs for the prevention of respiratory disease.

- 20 Typically, an agent capable of raising an immune response in a dog against *B. bronchiseptica* comprises inactivated or attenuated *B. bronchiseptica*, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Suitable agents that raise an immune response in a dog against *B.  
25 bronchiseptica* are known to a person of skill in the art. For example, the following dog vaccines are licensed for use.

COUGHGUARD-B<sup>®</sup> by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains an inactivated culture of *B. bronchiseptica*. It is for the immunisation of healthy dogs against disease caused by *B. bronchiseptica*, in particular kennel cough. COUGHGUARD-B<sup>®</sup> is prepared from a highly antigenic strain of *B. bronchiseptica* which has been inactivated and processed to be nontoxic when administered to dogs. The production method is reported to leave the immunogenic properties of *B. bronchiseptica* intact.

VANGUARD<sup>®</sup> 5/B by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains attenuated strains of canine distemper virus (CDV), CAV-2, CPV, and canine parvovirus (CPV) propagated on an established canine cell line. The CPV antigen was attenuated by low passage on the canine cell line and at that passage level has immunogenic properties capable of overriding maternal antibodies. The vaccine is packaged in lyophilised form with inert gas in place of vacuum. The bacterin component containing inactivated whole cultures of *B. bronchiseptica* which is supplied as diluent. The *B. bronchiseptica* component in VANGUARD<sup>®</sup> 5/B is prepared from a highly antigenic strain which has been inactivated and processed to be nontoxic when administered to dogs.

NASAGUARD-B<sup>™</sup> by Pfizer Animal Health (U.S. Vet. Lic. No.: 112) is composed of an avirulent live culture of *B. bronchiseptica* bacteria.

PROGARD<sup>®</sup>-KC by Intervet is a modified live intranasal vaccine containing attenuated canine parainfluenza virus and *Bordetella bronchiseptica* avirulent live culture. PROGARD<sup>®</sup>-KC is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD<sup>®</sup>-KC is for vaccination of healthy, susceptible puppies and dogs for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine parainfluenza virus and *B. bronchiseptica*.

PROGARD®-KC PLUS by Intervet contains live culture of avirulent strains of *B. bronchiseptica*, attenuated canine adenovirus type 2 and parainfluenza virus for intranasal administration. Vaccination with PROGARD®-KC Plus stimulates rapid, local immunity in the respiratory tract, thereby inhibiting infection at the port of entry as well as preventing clinical signs. In addition to local immunity, it also stimulates systemic immunity within three weeks of intranasal administration. The small volume (0.4 ml) and one nostril application of PROGARD®-KC Plus provide for ease in vaccination, particularly in small breeds and young puppies. PROGARD®-KC Plus is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD®-KC Plus is for vaccination of healthy dogs and puppies three weeks of age or older for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine adenovirus type 2, parainfluenza virus and *B. bronchiseptica*.

Intrac by Intervet is a freeze dried modified live vaccine, containing *B. bronchiseptica* strain S 55, for intranasal administration. Product licence number PL 0201/4011

Nobivac KC, described above, also contains *B. bronchiseptica*.

Vaccination would be useful especially but not exclusively for dogs prior to entry into a boarding kennel or for the vaccination of dogs in breeding facilities.

A typical dose of a vaccine comprised of recombinant protein is about 5-10 µg. A typical dose of a vaccine comprised of inactivated virus is about 1-10 mg.

In a ninth aspect, the invention provides the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or

(ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for stimulating an immune response against CRCV in a dog.

The invention includes the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for prophylaxis of respiratory disease in a dog, typically CIRD.

When a coronavirus protein, or an immunogenic fragment thereof, is used in the preparation of the medicament, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a BCV protein. Preferably the protein has

at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a CRCV protein.

Preferably, the coronaviral protein used in the preparation of the medicament is a BCV, HCV, HEV or CRCV protein, or a modification thereof, as described above with reference to the eighth aspect of the invention.

More preferably, the coronaviral protein used in the preparation of the medicament is an S protein. Yet more preferably, the S protein comprises an CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, or an immunogenic fragment thereof.

Most preferably, the coronaviral protein used in the preparation of the medicament comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Additionally or alternatively, the coronaviral protein used in the preparation of the medicament may comprise HE, E, M or N coronavirus proteins. In one embodiment, the HE, E, M or N proteins are BCV, HCV or HEV proteins. In another embodiment, the HE, E, M or N proteins are CRCV proteins.

Typically, the HE protein comprises an CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

- 5 Preferably, the coronaviral HE protein used in the preparation of the medicament comprises or consists of the partial amino acid sequence listed in Figure 14, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 14. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence  
10 listed in Figure 14. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the partial sequence listed in Figure 14.

- When a coronavirus is used in the preparation of the medicament, the  
15 coronavirus preferably comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the BCV S protein. More preferably the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the CRCV S protein.

- 20 Additionally or alternatively, the coronavirus may comprise an HE protein with at least 90%, or at least 95% amino acid identity with the BCV HE protein. More preferably the coronavirus comprises an HE protein with at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid identity with the CRCV HE protein.

- 25 In a tenth aspect, the invention provides a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the

methods of the fourth aspect of the invention, for use in medicine. Typically, the S protein will be used in veterinary medicine.

The invention includes a CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, for use in medicine. Typically, the HE protein will be used in veterinary medicine.

In an eleventh aspect, the invention provides a method of vaccinating a dog against CRCV, the method comprising administering to the dog a vaccine composition as described above in the ninth aspect of the invention.

Typically, the vaccine will be administered *via* the intramuscular, subcutaneous or intranasal routes

In another embodiment, a dog can passively acquire immunity against CRCV by being administered an antibody that reacts with CRCV. The antibody that reacts with CRCV may be an anti-BCV, anti-HCV antibody, but is preferably an anti-CRCV antibody. Preferably, the antibody that reacts with CRCV is an anti-S protein antibody an anti-HE protein antibody. Most preferably, the antibody that reacts with CRCV is an anti-CRCV S or HE protein antibody as described in the fifth aspect of the invention.

In a twelfth aspect, the invention provides a method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the methods as described above in the sixth aspect of the invention, or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, and, if the dog is infected with CRCV, quarantining the dog.

By "quarantining" a dog we include the meaning of keeping the dog separate from all other dogs. We also include the meaning of keeping the



dog separate from dogs that have not been vaccinated against CRCV, which can be performed as described above. We also include the meaning of keeping the dog separate from dogs that have not been infected by CRCV, which can be determined as described above.

- 5 In a thirteenth aspect, the invention provides a method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the methods described above in the sixth aspect of the invention, or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, and, if the dog is  
10 infected with CRCV, vaccinating other dogs that have been, are, or are likely to be in contact with the dog.

- A fourteenth aspect of the invention provides a method for identifying a test vaccine capable of preventing or reducing the incidence of canine infectious respiratory disease (CIRD) in dogs. The method comprises (a) determining  
15 whether a dog has been exposed to CRCV, typically according to the methods described above in the sixth aspect of the invention or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, (b) if the dog has not been exposed to CRCV, administering the test vaccine to the dog, (c) inoculating the dog with  
20 CRCV, and (d) determining whether the dog develops CIRD. The absence of CIRD in step (d) indicates that the test vaccine is capable of preventing CIRD.

Typically, this method is performed on a set of dogs.

- Preferably, the method involves the use of a set of control dog which are not  
25 administered the test vaccine in step (b). The significantly lower incidence of CIRD in the set of dogs that has been administered the test vaccine than

in the control set indicates that the test vaccine is capable of preventing or reducing the incidence of CIRD.

The invention also includes a vaccine identified by this method.

All of the documents referred to herein are incorporated herein, in their  
5 entirety, by reference.

The invention will now be described in more detail with the aid of the following Figures and Examples.

#### Figure 1

Partial nucleotide sequence (250 residues) of the CRCV polymerase (pol)  
10 cDNA (SEQ ID NO: 1).

#### Figure 2

Partial amino acid sequence (83 residues) of the CRCV pol protein (SEQ ID NO: 2), derived from the nucleotide sequence of Figure 1.

#### Figure 3

15 Nucleotide sequence (4092 residues) of the CRCV Spike (S) cDNA (SEQ ID NO: 3). The Y at position 3531 refers to either C or T.

#### Figure 4

Amino acid sequence (1363 residues) of the CRCV S protein (SEQ ID NO: 4), derived from the nucleotide sequence of Figure 3.

**Figure 5**

Consensus tree for cDNA sequences from a 250 nucleotide region of the polymerase gene of 12 coronaviruses. The sequence obtained from the canine respiratory coronavirus is designated T101. The numbers indicate  
5 bootstrap values obtained by analysis of 100 data sets.

BCV: bovine coronavirus, CCV: canine coronavirus, FIPV: feline infectious peritonitis virus, HEV: hemagglutinating encephalomyelitis virus, IBV: infectious bronchitis virus, MHV: mouse hepatitis virus, OC43: human coronavirus strain OC43, SDAV: sialodacryoadenitis virus, TCV: turkey  
10 coronavirus, TGEV: transmissible gastroenteritis virus, 229E: human coronavirus strain 229E, T101: canine respiratory coronavirus (PCR product from tracheal sample T101)

**Figure 6**

CLUSTAL X (1.8) multiple sequence alignment of the 250 nucleotide  
15 partial sequence of the pol cDNA of CRCV (sample T101, SEQ ID NO: 1), BCV (SEQ ID NO: 5), HCV strain OC43 (SEQ ID NO: 6), HEV (SEQ ID NO: 7) and CCV (enteric CCV, SEQ ID NO: 8).

**Figure 7**

CLUSTAL X (1.8) multiple sequence alignment of the 83 amino acid  
20 partial sequence of the pol protein of CRCV (protCRCVpol, SEQ ID NO: 2) with HCV (protHCVpoly, SEQ ID NO: 9), HEV (protHEVpoly, SEQ ID NO: 10), BCV (protBCVpoly, SEQ ID NO: 11) and CECV (enteric CCV, protCECVpol, SEQ ID NO: 12).

**Figure 8**

CLUSTAL X (1.8) sequence alignment of the nucleotide sequence of the CRCV spike cDNA (CRCVspike, SEQ ID NO: 3) and enteric CCV spike cDNA (CECVspike, SEQ ID NO: 13).

5 **Figure 9**

CLUSTAL X (1.8) multiple sequence alignment of the 4092 nucleotides of the CRCV spike cDNA (CRCVspike, SEQ ID NO: 3) sequence with BCV (BCVspike, SEQ ID NO: 14), HCV (HCVspike, SEQ ID NO: 15) and HEV (HEVspike, SEQ ID NO: 16) spike cDNAs. The Y at position 3531 in the  
10 CRCV sequence refers to either C or T.

**Figure 10**

CLUSTAL X (1.8) multiple sequence alignment of the 1363 amino acid sequence of the CRCV spike protein (CRCVspikepr, SEQ ID NO: 4) with BCV (BCVspikepro, SEQ ID NO: 17), HCV (HCVspikepro, SEQ ID NO: 18), HEV (HEVspikepro, SEQ ID NO: 19) and enteric CCV (CECVspikepr, SEQ ID NO: 20) spike proteins.  
15

**Figure 11**

RT-PCR using nested set of primers (Spike 1 and 2 (SEQ ID NOS: 34 and 35) followed by Spike 3 and 4 (SEQ ID NOS: 36 and 37)). BCV: Bovine coronavirus positive control sample; A72: Coronavirus negative A72 cells;  
20 H<sub>2</sub>O: PCR mix without DNA; T5 – T21: Tracheal samples of study dogs. The agarose gel electrophoresis shows PCR products of the expected size of 442bp for the positive control (BCV) and samples T12 and T21.

**Figure 12**

Comparison of the prevalence of respiratory disease in two groups of dogs. Dogs in group 1 were positive for serum antibodies to respiratory coronavirus on day of entry into the kennel, dogs in group 2 were negative.

5 The graph shows the percentage of dogs developing respiratory disease in group 1 compared to group2 ( $p < 0.001$ ).  $n$  is the total number of dogs in each group.

**Figure 13**

Partial nucleotide sequence (497 residues) of the CRCV hemagglutinin/esterase (HE) gene (SEQ ID NO: 21). The sequence corresponds to nucleotides 418 to 914 of the HE genes of BCV (GenBank M84486) and HCV OC43 (GenBank Accession No. M76373).

10

**Figure 14**

Partial amino acid sequence (165 residues) of the CRCV HE protein (SEQ ID NO: 22), derived from the nucleotide sequence of Figure 13. This sequence corresponds to amino acid residues 140 to 304 of BCV (GenBank M84486) and HCV OC43 (GenBank Accession No. M76373).

15

**Figure 15**

CLUSTAL X (1.8) multiple sequence alignment of a 497 nucleotide partial sequence of the hemagglutinin/esterase (HE) gene of CRCV (canine respiratory coronavirus, SEQ ID NO: 21) with BCV (bovine coronavirus strain LY138, (SEQ ID NO: 23, taken from Genbank Accession No. AF058942), OC43 (human coronavirus strain OC43, SEQ ID NO: 24 taken from Genbank Accession No. M76373), HECV (human enteric coronavirus, SEQ ID NO: 25, taken from Genbank Accession No. L07747), and HEV

20

25

(hemagglutinating encephalomyelitis virus, SEQ ID NO: 26, taken from Genbank Accession No. AF481863).

### Figure 16

CLUSTAL X (1.8) multiple sequence alignment of a 165 amino acid partial  
5 sequence of the HE protein of CRCV (canine respiratory coronavirus, (SEQ  
ID NO: 22) with BCV (bovine coronavirus strain LY138, SEQ ID NO: 27,  
taken from Genbank Accession No. AF058942), OC43 (human coronavirus  
strain OC43, SEQ ID NO: 28, taken from Genbank Accession No.  
M76373), HECV (human enteric coronavirus, SEQ ID NO: 29, taken from  
10 Genbank Accession No. L07747), and HEV (hemagglutinating  
encephalomyelitis virus, SEQ ID NO: 30, taken from Genbank Accession  
No. AF481863). The three CRCV-specific amino acids F, N and L are  
indicated in bold and are underlined.

### Figure 17

15 RT-PCR using consensus primers HE1 (SEQ ID NO: 38) and HE2 (SEQ ID  
NO: 39) directed to the HE gene of BCV and HCV (strain OC43). The  
agarose gel electrophoresis shows a PCR product of the expected size of  
497bp for the BCV positive control and for four tracheal samples from  
study dogs (T90, T91, T101 and T105), and not for coronavirus-negative  
20 A72 cells or the PCR mix without DNA (H<sub>2</sub>O). 1 kb indicates a molecular  
size standard (Promega).

### Figure 18

CRCV Spike gene cloning strategy.

## **Example 1: Detection of a novel coronavirus associated with canine infectious respiratory disease**

### **Summary**

An investigation into the causes of canine infectious respiratory disease (CIRD) was carried out in a large re-homing kennel. Tissue samples taken from the respiratory tract of diseased dogs were tested for the presence of coronaviruses using RT-PCR with conserved primers for the polymerase gene. Sequence analysis of four positive samples showed the presence of a novel coronavirus with high similarity to both bovine and human coronavirus (strain OC43) in their polymerase and spike genes whereas there was a low similarity to comparable genes in the enteric canine coronavirus. This canine respiratory coronavirus (CRCV) was detected by RT-PCR in 32/119 tracheal and 20/119 lung samples with the highest prevalence being detected in dogs with mild clinical symptoms. Serological analysis showed that the presence of antibodies against CRCV on the day of entry into the kennel decreased the risk of developing respiratory disease.

### **Materials and Methods**

#### **Study population**

Dogs from a well-established re-homing kennel with a history of endemic respiratory disease were monitored for this study. On entry into the kennel, all dogs were vaccinated with KAVAK DA<sub>2</sub> PiP69 (Fort Dodge) a live attenuated vaccine for distemper virus, canine adenovirus type 2, canine parainfluenzavirus and canine parvovirus. Also, a killed leptospirosis vaccine was used (Fort Dodge). The health status of each dog was assessed twice a day by a veterinary clinician and the respiratory symptoms were graded as follows: 1: no respiratory signs, 2: mild cough, 3: cough and nasal

discharge, 4: cough, nasal discharge and inappetence, 5: bronchopneumonia. The overall health status of the dogs was graded as follows: 1: good health, 2: poor health, 3: very poor health. The age, breed and sex of the dogs were recorded.

- 5 For 119 dogs a full post mortem examination was performed. The tissue samples were stored at  $-70^{\circ}\text{C}$  until further use.

Serum samples were collected from 111 dogs on day of entry into the rehoming kennel. For 81 dogs a follow-up serum was available on day 7 and for 111 dogs a serum was available on day 21 after entry.

- 10 Of the 111 dogs, 30 remained healthy during the 21 days between the first and the last serum sample whereas 81 dogs developed respiratory disease.

- Sera from 35 dogs housed elsewhere were obtained from the diagnostic service of the Royal Veterinary College. These sera had been submitted for biochemical analysis for various reasons. Five of these sera were from 18-month-old beagles with no history of respiratory disease. Sera were  
15 routinely stored at  $-20^{\circ}\text{C}$ .

#### RNA extraction and RT-PCR

- RNA was extracted from tracheal and lung tissue of 119 dogs using TriReagent (Sigma). Approximately 25-50 mg of homogenised tissue was  
20 used and RNA was extracted as recommended by the manufacturer.

Synthesis of cDNA was performed using Random Hexamers (Roche) and ImPromII reverse transcriptase (Promega).

The polymerase gene of coronaviruses is known to be highly conserved, and has previously been used for phylogenetic analysis of this virus family



(Stephensen *et al.*, 1999). For the detection of coronaviruses a modification of the primers 2Bp and 4Bm directed against the polymerase gene as described by Stephensen *et al.* (1999) were used (Conscoro5: 5' -ACT-CAR-ATG-AAT-TTG-AAA-TAT-GC (SEQ ID NO: 31); and Conscoro6: 5' -TCA-CAC-TTA-GGA-TAR-TCC-CA (SEQ ID NO: 32)).

PCR was performed using Taq polymerase (Promega) in the provided reaction buffer containing a final concentration of 2.5 mM MgCl<sub>2</sub> and 0.5 μM of primers. For PCR with the primers Conscoro5 and Conscoro6 the following temperature profile was used: After denaturation at 95°C for 5 min, 10 cycles were carried out at 95°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1 min. This was followed by 10 cycles using an annealing temperature of 45°C, 10 cycles at an annealing temperature of 50°C and 10 cycles at an annealing temperature of 53°C followed by a final extension at 72°C for 10 min.

A 20 μl fraction of the PCR product was analysed on a 1.5% agarose gel and blotted onto a nylon membrane (Roche) after electrophoresis. The nylon membrane was hybridised with an oligonucleotide probe specific for the PCR product at 37°C overnight (Probe Conscoro: AAG-TTT-TAT-GGY-GGY-TGG-GA (SEQ ID NO: 33)). The probe was 3'A-tailed with Digoxigenin-dUTP and was detected using anti-Digoxigenin conjugate and CSPD chemoluminescent substrate (Roche).

Primer sequences specific for the spike gene were derived from an alignment of the spike region of bovine coronavirus strain LY-138 (AF058942) and human coronavirus strain OC43 (L14643).

A PCR was performed with the primers Spike 1 and Spike 2, followed by a nested PCR using the primers Spike 3 and Spike 4 and 2 μl of the product of the first amplification.

The numbers in brackets refer to the nucleotide position in the bovine coronavirus genome.

Spike 1: 5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT (25291-25314)

Spike 2: 5'-CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG (25912-25890)

5 Spike 3: 5'-GTT-GGC-ATA-GGT-GAG-CAC-CTG (25320-25339)

Spike 4: 5'-GCA-ATG-CTG-GTT-CGG-AAG-AG (25762-25742)

Oligonucleotide Spike 1 has SEQ ID NO: 34, Spike 2 has SEQ ID NO: 35, Spike 3 has SEQ ID NO: 36, Spike 4 has SEQ ID NO: 37.

10 The temperature profile used was denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 40 sec and elongation at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The nested PCR produced a 442bp fragment.

15 PCR products were cloned into the pGEM-T-easy vector (Promega) and sequenced using the Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia) using Cy5 labelled primers.

### Phylogenetic analysis

20 An alignment of the 250 bp cDNA sequence from the polymerase gene to the corresponding sequences of 11 coronaviruses was performed using ClustalX (Thompson *et al.*, 1997).

The phylogenetic relationship to known coronaviruses was analysed using the Phylip 3.6 package (Felsenstein, 1989). The alignments were followed by a bootstrap analysis using the Seqboot programme. The obtained data sets were used for a maximum parsimony analysis using the DNAPars

programme and a consensus tree was calculated using Consense. The resulting trees were drawn using the Treeview programme (Page, 1996).

### ELISA

ELISA antigen for bovine coronavirus or enteric canine coronavirus (CECV) (the antigens are a preparation from virus infected cell cultures obtained from Churchill Applied Biosciences, Huntingdon, UK) was resuspended in PBS at the concentration recommended by the manufacturer and incubated on 96 well plates (Falcon) overnight at 37°C.

The plates were washed with PBS and blocked with PBS containing 5% skimmed milk powder for 30 min. The sera were diluted 1:100 in blocking buffer and incubated on the plates for 1h. After washing with PBS/ 0.05% Tween 20 (Sigma), a peroxidase labelled rabbit anti-dog IgG conjugate (Sigma) was added (1:5000 in PBS/0.05% Tween 20) for 1 h. The plates were incubated with colour substrate (OPD, Sigma) for 10 min and the reaction was stopped by adding 2M H<sub>2</sub>SO<sub>4</sub>. The adsorption was determined in an ELISA photometer at 492nm.

### Virus culture

Virus isolation is performed on canine adult lung fibroblasts (passage 3 to 7), MDCK and A72 cells. (It is appreciated, however, that virus isolation could be performed using primary cells or cell lines such as MDCK or A72 (canine), MDBK (bovine), HRT-18 (human rectal tumour cell line) and Vero (African Green Monkey). The lung fibroblasts are maintained in MEM with 20% fetal calf serum (FCS), MDCK and A72 cells are maintained in MEM with 5% FCS. Tracheal tissue samples (approx. 25mg) are homogenised using a scalpel and mixed vigorously in 1ml MEM containing Penicillin (100U/ml), Streptomycin (0.1mg/ml), Amphotericin B

(2.5µg/ml) and Trypsin (1µg/ml). The samples are centrifuged at 13000 rpm for 10 min. and the supernatant is used to inoculate cell cultures. After 30 min. at 37°C the supernatant is removed and maintenance medium added to the cultures. The cultures are passaged three times in the absence of a  
5 cytopathic effect. Then, RNA is extracted from the cells and RT-PCR to detect the presence of CRCV is performed.

### Statistical analysis

The data were analysed using the chi-square test or Fisher's exact test and p values below 0.05 were considered statistically significant.

## 10 Results

### PCR using consensus primers for the coronavirus RNA polymerase gene

Using the primers Conscoro5 and Conscoro6, cDNA obtained from 40 tracheal samples was analysed by RT-PCR.

15 Out of these, seven were found to be positive by PCR and subsequent hybridisation (17.5%).

The PCR products were cloned and sequenced (Figures 1 and 2) and the sequence data were compared to available viral sequences using the FASTA search program (Pearson, 1990).

20 Comparison of the coronavirus cDNA polymerase sequence obtained from four of the canine tracheal samples to other coronavirus sequences revealed that they were most similar to sequence data from BCV strain Quebec and LY138 (Genbank Accession Nos. AF220295 and AF058942, respectively) and human coronavirus strain OC43 (Genbank Accession No. AF124989).

The similarity in the analysed 250 bp sequence was 98.8% for BCV Quebec, and 98.4% for BCV LY138 and the HCV pol genes, whereas it was only 68.53% for CCV strain 1-71 pol gene (Figures 6 and 7).

5 An alignment of the novel sequence with the corresponding sequences of 11 coronaviruses and phylogenetic analysis using the maximum parsimony method resulted in the consensus tree shown in Figure 5. The cDNA sequence obtained from a tracheal sample (T101) was found on a common branch with bovine coronavirus, human coronavirus-OC43 and hemagglutinating encephalomyelitis virus.

10 The virus was called canine respiratory coronavirus (CRCV).

#### PCR using primers for the spike gene

For further analysis of the RNA sequence of CRCV, an alignment of the RNA for the spike gene of the bovine coronavirus LY 138 strain (AF058942) and the human coronavirus OC43 strain (L14643) was  
15 performed using Clustal X (Thompson *et al.*, 1997). Consensus regions were chosen for the selection of the nested primer sets Spike 1-2 and Spike 3-4 (Figure 11). PCR analysis was performed with the cDNA obtained from 119 tracheal and lung samples using these nested primers.

In total 32 tracheal samples (26.9%) and 20 lung samples (16.8%) were  
20 found positive by nested PCR. For eight dogs a positive PCR result was obtained for both, trachea and lung.

Sequence analysis of the PCR products obtained from tissues of six different dogs showed identical DNA sequences for these cDNAs (Figures 3 and 4). A comparison to known coronavirus spike sequences using the  
25 FASTA program revealed a 98.1% similarity to bovine coronavirus and a 97.8% similarity to human coronavirus OC43 (Figures 9 and 10).

### PCR using primers for the HE gene

Bovine coronavirus and other group II coronaviruses contain an additional structural protein, the hemagglutinin/esterase (HE). Because of the high similarity of CRCV with BCV, we analysed the presence of an HE gene in  
5 CRCV.

An alignment of the HE genes sequences of BCV and HCV OC43 was used to design the primers HE1 and HE2 (Table 2). Four tracheal samples that had previously been identified as positive for coronavirus RNA by RT-PCR with primers for the S gene were tested by RT-PCR with the primer set for  
10 the HE gene. All four samples showed a PCR band of the expected size after agarose gel electrophoresis (Figure 17).

**Table 2: Primers designed from an alignment of the hemagglutinin/esterase genes of BCV (GenBank Accession No. M84486) and HCV OC43 (GenBank Accession No. M76373)**

15

Name	Sequence	Location in BCV HE gene
HE 1	5'-TAT-CGC-AGC-CTT-ACT-TTT-GT	418-437
HE 2	5'-ACC-GCC-GTC-ATG-TTA-TCA-G	914-896

Primer HE1 has SEQ ID No: 38 and HE2 has SEQ ID No: 39. The sequence of the CRCV PCR product obtained using primers HE 1 and HE 2 is given in Figure 13 (SEQ ID No: 21), and its predicted amino acid  
20 sequence is listed in Figure 14 (SEQ ID No: 22). A comparison of these nucleotide and amino acid sequences with the corresponding fragments of other related coronaviruses is shown in Figures 15 and 16. Three amino acids were shown to be unique to CRCV, as shown in Table 3.

**Table 3: Unique amino acids in CRCV HE gene**

Amino acid in CRCV	Amino acid in BCV/ HECV/ HCV/ HEV	Position in BCV/ HECV/ HCV/ HEV	Position in PCR product HE1-HE2
F (Phe)	L (Leu)	235	96
N (Asn)	T (Thr)	242	103
L (Leu)	V (Val)	253	114

The amino acid positions in BCV, HECV, HCV and HEV are numbered from the initial M (which is number 1) at the start of the BCV and HCV OC43 HE proteins (GenBank Accession Nos. M84486 and M76373, respectively).

#### 5 Association of PCR positive samples with respiratory signs

Using primers for the spike gene, tracheal and lung samples from 119 dogs were analysed by RT-PCR for CRCV. Of these 42 were from dogs with no respiratory signs (grade 1), 18 dogs had shown mild respiratory signs (grade 2), 46 had shown moderate (grade 3) and 13 severe respiratory signs (grades 4 and 5). Grades 4 and 5 were merged due to the low case numbers in these groups.

Table 4 shows the PCR results for coronavirus in dogs with different grades of respiratory disease. Specifically, Table 4 shows the RT-PCR results from tracheal and lung samples of 119 dogs with different respiratory signs (none to severe) using a nested PCR directed against the coronavirus spike gene as well as the number of positive samples out of total sample number and the percentage of positive samples (in brackets).

**Table 4: RT-PCR results for tracheal and lung samples**

Respiratory signs	Trachea: Positive samples	Lung Positive samples	Trachea and lung Positive samples
None	11/42 (26.2%)	8/42 (19.1%)	2/42
Mild	10/18 (55.6%)	4/18 (22.2%)	4/18
Moderate	9/46 (19.6%)	8/46 (17.4%)	2/46
Severe	2/13 (15.4%)	0/13	0/13

**Establishment of a serological assay for CRCV**

Because of the homology of the spike cDNA of CRCV to the spike region  
 5 of bovine coronavirus, an ELISA antigen for BCV was used for serological  
 analysis of CRCV.

Sera from five dogs with no history of infectious respiratory disease that  
 had not been housed in the investigated kennel were tested. The OD values  
 ranged from -0.013 to 0.39 with an average OD value of 0.154.  
 10 Furthermore, sera from 30 dogs admitted to a veterinary clinic for various  
 reasons were tested for antibodies to coronavirus. Of these, 20 samples  
 showed an OD of <0.4 (-0.46 to 0.396) and 10 samples showed an OD of  
 >1.0 (1.012 to 1.949). Samples with an OD of 0.6 or above were  
 subsequently considered positive.



### Comparison of the immune response to CRCV of dogs with and without respiratory disease

The BCV-antigen ELISA was performed using paired sera of 111 dogs from the study kennel. Of these, 81 dogs had shown symptoms of respiratory disease during a period of 21 days and 30 had remained healthy.

Of the group of dogs with respiratory disease, 17 were positive for antibodies to CRCV on the day of entry into the kennel and 64 were negative.

Of the 64 dogs with no detectable antibodies to BCV on day one, 63 tested positive on day 21. All 46 dogs out of these 63 for which a sample on day 7 was available tested negative on day 7. Therefore 63 dogs showed a seroconversion during the study-period whereas only one dog remained negative.

Of the 31 dogs that had remained healthy, 17 had antibodies to CRCV on the day of entry. All of the 13 dogs that were negative on day 1 tested negative on day 7 but showed a seroconversion by day 21.

Thus, of 34 dogs that were positive for antibodies to CRCV on arrival in the kennel, 17 developed respiratory disease (50%) whereas of 77 dogs that were negative on arrival, 64 developed respiratory signs during the study-period (83.1%), (Figure 12).

Therefore dogs that had no antibodies to CRCV on entry into the kennel had an increased probability of developing respiratory disease ( $p < 0.001$ ).

Only one out of the 77 dogs that were negative on arrival remained negative during the study period of 21 days whereas 76 dogs showed a seroconversion.

### Serology using canine enteric coronavirus (CECV) antigen

An ELISA assay using a canine coronavirus antigen was performed to investigate whether CRCV showed a serological cross reaction to canine enteric coronavirus. Sera from 27 dogs, previously tested for antibodies to CRCV using the BCV antigen were selected.

It was found that eight dogs had antibodies to CECV on the day of entry into the kennel, of these four also had antibodies to CRCV. Nineteen dogs were found to be negative for CECV on day 1, 17 of these were also negative for CRCV. Of the 19 negative dogs, five showed a seroconversion to CECV during the 21-day period of the investigation and 17 showed a seroconversion to CRCV.

Analysis of the prevalence of respiratory disease in this group showed that six out of the eight dogs (75%) that were positive for antibodies to CECV on day 1 developed respiratory disease. Out of the group of 19 dogs that had no detectable antibodies to CECV on day 1, 15 showed signs of respiratory disease (78.9%), ( $p=0.594$ ).

### Virus isolation

Tracheal tissue samples from dogs that are identified as positive for CRCV RNA by RT-PCR are inoculated on cell cultures of canine adult lung fibroblasts and MDCK cells. For some samples, virus isolation is also performed on A72 cells. The cultures show no signs of a cytopathic effect during three passages. After several passage, RNA is extracted from the cultures and tested for the presence of CRCV RNA by RT-PCR.

## Discussion

This study reports the detection of a novel coronavirus, CRCV, in kennelled dogs with respiratory disease.

Coronaviruses have been reported to cause respiratory disease of man, cattle, swine and poultry, but their presence in the respiratory tract of dogs and a possible association with canine infectious respiratory disease (CIRD) has not been determined.

Dogs were investigated from a kennel in which CIRD was endemic and could not be controlled by the use of vaccines recommended against CIRD. Samples taken from the respiratory tract of these dogs were examined using RT-PCR primers directed to the conserved polymerase gene of coronaviruses (Stephensen *et al.*, 1999).

Initially, seven tracheal samples were found to be positive; the sequence of the RT-PCR products was determined and compared to all available coronavirus polymerase gene sequences. This analysis revealed that the cDNA sequence obtained from the canine samples had the highest similarity to the polymerase gene of bovine coronavirus (98.8%) and human coronavirus OC43 (98.4%) but only a very low similarity to the polymerase gene of the enteric canine coronavirus (strain 1-71, 68.53% similarity).

A phylogenetic analysis was performed using the polymerase sequences of eleven additional coronaviruses. The coronavirus detected in the respiratory tract of dogs (CRCV) was located on a common branch with three group 2 viruses: BCV, HCV strain OC43 and HEV. However, canine enteric coronavirus, a group 1 coronavirus, was shown to be only distantly related.

Canine respiratory coronavirus therefore is a novel coronavirus of dogs that is most closely related to BCV and HCV-OC43, both of which are known to cause respiratory disease.

To obtain more sequence information and to further determine the relationship to other coronaviruses using a more variable gene, a part of the spike gene was analysed. Since CRCV had been shown to be most similar to BCV and HCV-OC43, an alignment of the sequences of their spike genes was used to design a nested set of primers. Nested primers were chosen to achieve a more sensitive assay.

Sequencing of the products of this RT-PCR confirmed the high similarity of CRCV with BCV and HCV-OC43.

The presence of antibodies to CRCV was analysed using an ELISA based on a BCV antigen because of the high sequence similarity of the two viruses in the spike cDNA. The ELISA results confirmed the presence of a virus similar to BCV in the study population.

The prevalence of antibodies was 30% at the time of entry into the kennel and 99% after 21 days.

Interestingly and unexpectedly, serological analysis revealed that dogs with antibodies to CRCV on day of entry into the kennel developed respiratory disease less frequently than dogs without antibodies ( $p < 0.001$ ). Therefore the presence of antibodies to CRCV had a protective effect against respiratory disease in this population.

Almost all dogs negative on day of entry into the kennel showed a seroconversion to CRCV within three weeks, indicating that the virus is highly contagious. Serology using an antigen for canine enteric coronavirus (CECV) showed a much lower prevalence of antibodies to CECV on day

21. Therefore the BCV-ELISA results did not reflect an infection with canine enteric coronavirus and the cross-reactivity between the two antigens seems to be low.

Serum antibodies to CRCV were present in about 30% of dogs of various origins including dogs entering a re-homing kennel as well as pet dogs. The presence of CRCV is therefore not limited to the investigated kennel and the virus seems to be established in the dog population.

By PCR, CRCV was detected in tracheal tissue and lung tissue and therefore appears to infect the upper and lower respiratory tract of dogs. Within the kennelled population, CRCV-RNA was detected in 27.3% of dogs with all grades of respiratory disease as well as in 26.2% of dogs that were apparently healthy at the time of euthanasia.

CRCV-RNA was most frequently found in the trachea of dogs with mild cough (55%). Studies using the human coronavirus strain 229E have shown, that coronaviruses can cause disruption of the respiratory epithelium and ciliary dyskinesia (Chilvers *et al.*, 2001). Without being bound by theory, we believe that an infection with CRCV has a similar effect, and that the virus plays an important role in the early stages of the pathogenesis of CIRD. By damaging the respiratory epithelium and disrupting ciliary clearance CRCV facilitates the entry of other viral or bacterial pathogens. Therefore while CRCV infection on its own may cause only mild respiratory symptoms, in conjunction with other pathogenic agents it could lead to severe respiratory disease.

The pathogenesis of CIRD has not been thoroughly investigated since the 1970s when *Bordetella bronchiseptica*, canine adenovirus type 2 and canine parainfluenza were determined to be the main causes of the disease. However the vaccination of all dogs against CPiV, CAV-2 and distemper

virus did not help to control the disease in this kennel despite evidence that the majority of dogs responded to the vaccine within 21 days (data not shown).

This study shows an association of a novel canine respiratory coronavirus with CIRD. The aetiology of CIRD therefore needs to be re-evaluated and the role of novel microorganisms or microorganisms previously not associated with the disease has to be established.

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**Example 2: Cloning and expression of CRCV Spike**

The CRCV Spike gene was cloned using the primers listed in Table 5 and using the following cloning strategy, which is illustrated in Figure 18.

1. The spike gene was amplified in four overlapping fragments (A,B,C,D).
2. The PCR product Sp5-Sp2 (B) was joined to the product Sp1-Sp8 (C) using the *PvuII* site in the overlap.
3. This fragment was cloned into the pT7blue2 vector (Novagen) using the restriction sites *NcoI* and *BstXI*.
4. The PCR fragment SpFXho-Sp6 (A) was joined to BC using the restriction site *BstXI* in the overlap and the *XhoI* site that had been incorporated into the primer SpF-Xho.
5. Fragment ABC was moved into the baculovirus transfer vector pMelBacB (Invitrogen) using the restriction sites *XhoI* and *NcoI*.
6. The PCR fragment Sp7-SpR-HisTag- Eco (D) was joined to ABC using the restriction site *NcoI* in the overlap and the *EcoRI* site that had been incorporated into the primer SpR-Eco-HisTag resulting in the complete spike gene in pMelBacB (Spike MelBac). This construct contains a HisTag (6xHis) at the C terminus of the expressed protein.
7. For mammalian expression the complete gene was moved to pSecTagA (Invitrogen) using the *BamHI* site in pMelBacB and the *EcoRI* site at the end of ABCD resulting in the plasmid SpikeSecTag.

### Construction of a recombinant baculovirus

A co-transfection was performed in Sf9 cells using the Bac-N-Blue baculovirus DNA (Invitrogen) and Spike MelBac. The resulting baculovirus (AcSpCRCV 1-11) was shown to contain a full-length insert by  
5 PCR using primers (Invitrogen) located upstream and downstream of the recombination site.

### Expression in mammalian cells

The plasmid Spike SecTag was transfected into BHK-21 cells using Lipofectamine (Invitrogen). Expression of the Spike protein was analysed  
10 using a serum sample from a dog that had been shown to be positive for antibodies to CRCV using ELISA (BCV antigen obtained from Churchill) and a positive control serum for BCV obtained from Churchill (chicken anti BCV). The transfected cells showed a positive signal in an immunofluorescence assay using the canine or the chicken serum and a  
15 FITC labelled conjugate (FITC anti-dog IgG or FITC anti Chicken IgG).

SpF-XhoI contains a Xho I site (**bold**). SpR-His-EcoR I contains a 6xHisTag (double-underlined), a stop codon (underlined) and an EcoR I site (**bold**)

Name	Sequence	SEQ ID NO:	Location in BCV spike gene
Sp 1	5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT	40	1637-1660
Sp 2	5'-CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG	41	2258-2236
Sp 3	5'-GTT-GGC-ATA-GGT-GAG-CAC-TG	42	1666-1686
Sp 4	5'-GCA-ATG-CTG-GTT-CGG-AAG-AG	43	2107-2088
Sp 5	5'-AAC-GGT-TAC-ACT-GTT-CAG-CC	44	931-950
Sp 6	5'-CAA-GTA-AAT-GAG-ICT-GCC-TG	45	1121-1102
Sp 7	5'-GGC-TGC-CAC-CTC-TGC-TAG-TC	46	2919-2938
Sp 8	5'-ATT-GTT-AAA-TGC-ATT-AGC-AAT-AAG-C	47	3069-3045
SpF	5'-TTT-TTG-ATA-CTT-TTA-ATT-TCC-TTA-CC	48	4-29
SpR	5'-GTC-GTC-ATG-TGA-WGT-TTT-RAT-TAC	49	4089-4066
SpF- <i>Xho</i> I	5'-AGC-TCG-AGC-TTT-TTG-ATA-CTT-TTA-ATT-TCC-TTA-CC	50	
SpR His- <i>Eco</i> RI	5'-TTG-AAT-ICT-TAA-TGA-TGA-TGA-TGG-TGG-TCA-TGT-GAW-GTT-TTR-AIT-AC	51	

CLAIMS

1. A coronavirus Spike (S) protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein whose amino acid sequence is listed in Figure 4, and comprising at least one of the canine respiratory coronavirus (CRCV)-specific amino acids listed in Table 1.  
5
2. A coronavirus S protein that comprises the amino acid sequence listed in Figure 4, or a variant thereof with at least 97% amino acid sequence identity with the sequence listed in Figure 4.  
10
3. A coronavirus polymerase (pol) protein, or fragment thereof, having at least 90% amino acid sequence identity with BCV pol protein and comprising the amino acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).  
15
4. A coronavirus pol protein that comprises the amino acid sequence listed in Figure 2.
- 20 5. A coronavirus hemagglutinin/esterase (HE) protein, or fragment thereof, having at least 90% amino acid sequence identity with BCV HE protein and comprising one or more of F at position 235, N at position 242 and L at position 253 of the BCV HE protein (Genbank Accession No. AF058942).  
25
6. A coronavirus HE protein that comprises the amino acid sequence listed in Figure 14.

7. A polynucleotide that encodes the protein according to any of Claims 1 to 6, or the complement thereof.
8. A polynucleotide according to Claim 7 comprising the nucleotide  
5 sequence listed in Figure 3.
9. A polynucleotide according to Claim 7 comprising the nucleotide sequence listed in Figure 1.
10. A polynucleotide according to Claim 7 comprising the nucleotide  
10 sequence listed in Figure 13.
11. A vector comprising the polynucleotide of any of Claims 7 to 10.
12. A host cell comprising the vector of Claim 11.  
15
13. A method of obtaining a protein encoded by the vector of Claim 11, the method comprising culturing the host cell of Claim 12, expressing the protein in the host cell, and purifying the protein.  
20
14. A protein obtainable by the method of Claim 13.
15. A host cell according to Claim 12 wherein the vector is an expression vector comprising a eukaryotic promoter operatively linked to the  
25 polynucleotide, and wherein the host cell is a eukaryotic host cell.
16. A method of obtaining a glycosylated S protein encoded by the polynucleotide of Claim 7 or 8, the method comprising culturing the host cell of Claim 15, expressing the protein in the host cell, and purifying the  
30 protein.

17. A glycosylated S protein obtainable by the method of Claim 16.
18. A method of making an anti-CRCV antibody comprising (i) raising  
5 an immune response to an S protein according to any of Claims 1, 2, 14 or  
17 in an animal and preparing an antibody from the animal or from an  
immortal cell derived therefrom, or (ii) selecting an antibody from an  
antibody-display library using an S protein according to any of Claims 1, 2,  
14 or 17.
- 10 19. A method according to Claim 18 further comprising determining  
whether the antibody has greater affinity for the CRCV S protein than for  
the BCV S protein.
- 15 20. An anti-CRCV antibody obtainable by the method of Claim 18 or 19  
, that has greater affinity for the CRCV S protein than for the BCV S  
protein.
21. A method of making an anti-CRCV antibody comprising (i) raising  
20 an immune response to an HE protein according to Claim 5 or 6 in an  
animal and preparing an antibody from the animal or from an immortal cell  
derived therefrom, or (ii) selecting an antibody from an antibody-display  
library using an HE protein according to Claim 5 or 6.
- 25 22. A method according to Claim 21 further comprising determining  
whether the antibody has greater affinity for the CRCV HE protein than for  
the BCV HE protein.

23. An anti-CRCV antibody obtainable by the method of Claim 21 or 22, that has greater affinity for the CRCV HE protein than for the BCV HE protein.
- 5 24. A method of determining whether a dog has been exposed to CRCV, the method comprising:
- (a) obtaining a suitable sample from the dog; and
  - (b) identifying CRCV or an anti-CRCV antibody in the sample.
- 10 25. A method according to Claim 24 or 25 wherein the anti-CRCV antibody is detected using a CRCV, BCV, human coronavirus (HCV) or hemagglutinating encephalomyelitis virus (HEV) antigen.
- 15 26. A method according to Claim 25 wherein the suitable sample is an antibody containing sample such as serum, saliva, tracheal wash and bronchiolar lavage, and wherein identifying an anti-CRCV antibody comprises identifying an antibody that selectively binds to BCV S protein (AF058942) HCV S protein (L14643), or to a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein (Figure
- 20 4) or a fragment thereof.
27. A method according to Claim 25 wherein identifying an anti-CRCV antibody comprises identifying an antibody that selectively binds to BCV HE protein (AF058942) HCV HE protein (M76373), or to a coronavirus
- 25 having an HE protein with at least 90% amino acid identity with CRCV HE protein (Figure 14) or a fragment thereof.
28. A method according to Claim 24 wherein the suitable sample is an antibody containing sample, and wherein identifying an anti-CRCV
- 30 antibody comprises identifying an antibody that selectively binds to an S

protein according to any of Claims 1, 2, 11 or 14 or to an HE protein according to Claim 5 or 6.

29. A method according to Claim 24 wherein the suitable sample is a lung wash, tracheal wash, tonsillar swab or a biopsy or post-mortem sample from the respiratory tract of the dog.

30. A method according to Claim 29 wherein identifying CRCV comprises identifying a nucleic acid component of CRCV.

10

31. A method according to Claim 30 wherein identifying a nucleic acid component of CRCV comprises identifying a polynucleotide that hybridises at high stringency to the BCV genome (AF058942).

32. A method according to Claim 30 or 31 wherein identifying CRCV comprises identifying a polynucleotide as defined in any of Claims 7 to 10.

33. A method according to Claim 29 wherein identifying CRCV comprises identifying a protein component of CRCV.

20

34. A method according to Claim 33 wherein identifying a protein component of CRCV comprises identifying a protein according to any of Claims 1, 2, 5, 6, 11 or 14.

35. A method according to Claim 33 or 34 wherein identifying a protein component of CRCV comprises using an antibody reactive with CRCV.

36. A method according to Claim 35 wherein the antibody reactive with CRCV is an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody according to Claim 20 or 23.

30



37. An immunosorbent assay for detecting anti-CRCV S antibodies, the assay comprising:

a solid phase coated with an S protein according to any of Claims 1, 2, 14 or 17, or an antigenic fragment thereof, wherein anti-CRCV S antibodies in a sample exposed to the solid phase will bind to the protein; and

a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

10

38. An immunosorbent assay for detecting anti-CRCV HE antibodies, the assay comprising:

a solid phase coated with an HE protein according to Claim 5 or 6, or an antigenic fragment thereof, wherein anti-CRCV HE antibodies in a sample exposed to the solid phase will bind to the protein; and

a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

39. An immunosorbent assay according to Claim 37 or 38, wherein the solid phase is a microtitre well.

40. An immunosorbent assay according to Claim 37 or 38, wherein the conjugate comprises anti-dog antibody.

41. An immunosorbent assay according to any of Claims 37 to 40, wherein the conjugate comprises an enzyme.

42. An immunosorbent assay according to Claim 41, wherein the enzyme is horseradish peroxidase.

30

43. An immunosorbent assay according to Claim 41 or 42, further comprising a substrate for the enzyme.
44. A solid substrate with an S protein according to any of Claims 1, 2,  
5 14 or 17, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV S antibodies from a liquid sample, wherein anti-CRCV S antibodies in a sample exposed to the solid substrate will bind to the S protein.
- 10 45. A solid substrate with an HE protein according to Claim 5 or 6, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV HE antibodies from a liquid sample, wherein anti-CRCV HE antibodies in a sample exposed to the solid substrate will bind to the HE protein.
- 15 46. A solid substrate according to Claim 44 or 45, wherein the solid substrate is a microtitre well.
47. A vaccine composition for vaccinating dogs comprising a coronavirus having an S protein with at 75% least amino acid identity with  
20 BCV S protein, or a coronaviral protein having at least 75% amino acid identity with a BCV protein or an immunogenic fragment thereof, or a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof.
- 25 48. A vaccine composition according to Claim 47 wherein the coronaviral protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.
49. A vaccine composition according to Claim 47 or 48 wherein the  
30 coronaviral protein is an S protein.

50. A vaccine composition according to Claim 49 comprising an S protein according to any of Claims 1, 2, 14 or 17, a BCV S protein, an HCV S protein, an HEV S protein, or an immunogenic fragment thereof.

5

51. A vaccine composition according to Claim 47 or 48 wherein the coronaviral protein is a hemagglutinin-esterase protein (HE) or an integral membrane protein (M).

10 52. A vaccine composition according to Claim 51 wherein the HE protein comprises an HE protein as defined in Claim 5 or 6, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

15 53. A vaccine composition according to Claim 47 wherein the virus is selected from BCV, HCV, HEV and CRCV, or a modification thereof.

54. A vaccine composition according to any of Claims 47 to 53 and also comprising a pharmaceutically acceptable adjuvant.

20 55. A vaccine composition according to any of Claims 47 to 55 further comprising any one or more of:

(a) an agent capable of raising an immune response in a dog against canine parainfluenza virus (CPIV);

25 (b) an agent capable of raising an immune response in a dog against canine adenovirus type 2 (CAV-2);

(c) an agent capable of raising an immune response in a dog against canine herpesvirus (CHV); and

(d) an agent capable of raising an immune response in a dog against *Bordetella bronchiseptica* (*B. bronchiseptica*).

56. Use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for stimulating an immune response against CRCV in a dog.

57. Use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for prophylaxis of respiratory disease in a dog.

58. Use according to Claim 56 or 57 wherein the coronaviral protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

59. Use according to any of Claims 56 to 58 wherein the coronaviral protein is an S protein.
60. Use according to Claim 59 wherein the S protein comprises an S protein according to any of Claims 1, 2, 14 or 17, a BCV S protein, an HCV S protein, or an immunogenic fragment thereof.
61. Use according to any of Claims 56 to 58 wherein the coronaviral protein is HE or M.
62. Use according to Claim 61 wherein the HE protein comprises an HE protein according to Claim 5 or 6, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.
63. Use according to Claim 56 wherein the virus is selected from BCV, HCV, HEV and CRCV, or a modification thereof.
64. An S protein according to any of Claims 1, 2, 14 or 17 for use in medicine.
65. An HE protein according to Claim 5 or 6 for use in medicine.
66. A method of vaccinating a dog against CRCV, the method comprising administering to the dog a vaccine composition according to any of Claims 47 to 54.
67. A method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the method of any of Claims 24 to 36 and, if the dog is infected with CRCV, quarantining the dog.

68. A method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the method of any of Claims 24 to 36 and, if the dog is infected with CRCV,  
5 vaccinating other dogs that have been, are, or are likely to be in contact with the dog.

69. A method for identifying a test vaccine capable of preventing canine infectious respiratory disease (CIRD) in dogs, comprising  
10 (a) determining whether a dog has been exposed to CRCV according to the method of any of Claims 24 to 36,  
(b) if the dog has not been exposed to CRCV, administering the test vaccine to the dog,  
(c) inoculating the dog with CRCV, and  
15 (d) determining whether the dog develops CIRD,  
wherein the absence of CIRD in step (d) indicates that the test vaccine is capable of preventing CIRD.

70. A vaccine identified by the method of Claim 69.  
20

71. The *E. coli* strain Spike D-1 CRCV, containing a plasmid whose insert contains a portion of the CRCV S cDNA, as deposited by the Royal Veterinary College at the NCIMB under Accession number NCIMB 41146 on 25 July 2002.

25 72. The plasmid contained in *E. coli* strain Spike D-1 CRCV, deposited by the Royal Veterinary College at the NCIMB under Accession number NCIMB 41146 on 25 July 2002.

73. A kit of parts for the immunosorbent assay according to any of Claims 37-43, comprising a solid phase, a CRCV or CRCV-like S protein and/or a CRCV or CRCV-like HE protein for coating the solid phase, and a detectable label conjugate.

5

74. A kit of parts according to Claim 73 wherein the solid phase comprises a microtitre plate.

75. A kit of parts according to Claim 73 or 74 wherein the detectable  
10 label conjugate comprises an anti-dog antibody.

76. A kit of parts according to Claim 73 or 74 wherein the detectable label conjugate comprises an enzyme.

15 77. A kit of parts according to Claim 76 further comprising a substrate for the enzyme.

78. A kit of parts according to any of Claims 73 to 77 further comprising a positive control sample that contains an anti-CRCV S protein antibody  
20 and/or an anti-CRCV HE protein antibody.

79. A method of passively immunising a dog against CRCV, comprising administering an antibody that reacts with CRCV to the dog.

80. A method according to Claim 79 wherein the antibody that reacts  
25 with CRCV comprises an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody.

81. A method according to Claim 79 or 80 wherein the antibody that reacts with CRCV comprises an anti-S protein antibody or an anti-HE protein antibody.

82. A method according to any of Claims 79 to 81 wherein the antibody  
5 that reacts with CRCV comprises the anti-CRCV antibody according to Claim 20 or 23.

83. Use of an antibody that reacts with CRCV in the preparation of a medicament for passively immunising a dog against CRCV.

84. Use according to Claim 83 wherein the antibody that reacts with  
10 CRCV is an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody.

85. Use according to Claim 83 or 84 wherein the antibody that reacts with CRCV is an anti-S protein antibody or an anti-HE protein antibody.

86. Use according to any of Claims 83 to 85 wherein the antibody that  
15 reacts with CRCV comprises the anti-CRCV antibody according to Claim 20 or 23.

87. A method of diagnosing CIRD the method comprising determining whether a dog has been exposed to CRCV according to the method defined in any one of Claims 24 to 36.



FIGURE 1 *K*

```
ctcagatgaa tttgaaatat gctattagtg ctaagaatag agcccgcact gttgctgggtg 60
tttcataact tagtactatg actggcagaa tgtttcatca aaaatgtttg aaaagtatatg 120
cagctacacg tgggtgttcct gttgttatag gcaccactaa attttatggc ggctgggatg 180
atatgttacg tcgccttatt aaagatgttg acaatcctgt acttatgggt tgggattatc 240
ctaagtgtga 250
```

## FIGURE 2

```
QMNLKYAISA KNRARTVAGV SILSTMTGRM FHQKCLKSIA ATRGVPVVIG TTKFYGGWDD 60
MLRRLIKDVE NPVLMGWDYP KCE 84
```

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## FIGURE 3 (Page 1 of 2)

atgtttttga tacttttaat ttccttacca atggcttttg ctgttatagg agattttaaag	60
tgtactacgg tttccatcaa tgatgttgac accggtgctc cttctattag cactgatgtt	120
gtcgaagtta ctaatgggtt aggtacttat tatgttttag atcgtgtgta tttaaatact	180
acattgtttg ttaatgggtt ttatcctact tcagggttcta catatcgtaa tatggcactg	240
aagggaactt tactattgag cacactatgg tttaaaccac catttctttc tgattttatt	300
gatgggtgtt ttgctaaggt aaaaaatacc aaggttatta aagatgggtg agtgatatgt	360
gagtttctcg ctataactat aggtagtact tttgtaaata catcctatag tgtggtagta	420
caaccacata ctactaatit agataataaa ttacaagggtc tcttagagat ctctgtttgc	480
cagtatacta tgtgcgatta cccacatacg atgtgtcctc ctaatctggg taataaacgc	540
atagaactat ggcattggga tacagggtgt gttccctgtt tatataagcg taatttcaca	600
tatgatgtga atgctgatta tttgtattcc catttttctc aagaagggtg tactttttat	660
gcataattta cagacactgg tgttgttact aagtttctgt ttcattgtta tttaggcacg	720
gtgctttcac attattatgt catgcccttg acttgtaata gtgctatgac tttagaatac	780
tgggttacac ctctcacttt taaacaatat ttactcgctt tcaatcaaga tgggtgttatt	840
tttaatgctg ttgattgtaa gagtgatttt atgagtgaga ttaagtgtaa aacactatct	900
atagcaccat ctactgggtt ttatgaatta aacgggtaca ctgttcagcc aattgcagat	960
gtttaccgac gtatacctaa tcttcccgat tgtaatatag aggcttggct taatgataag	1020
tcgggtgcctt ctccattaaa ttgggaacgt aagacctttt caaattgtaa ttttaatatg	1080
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aagcctcaac ctgtaggtgt ttttactgat catgatgttg tttatgcaca acattgtttt	1440
aaagctccca caaatttctg tccgtgtaaa ttgaatgggt ctttgtgtgt aggtagtggg	1500
tttgggtatg atgctgggtt taaaaatagt ggtataggca cttgtcctgc aggtactaat	1560
tattttaactt gttataatgc taaccaatgt gattgtttgt gcaactccaga ccctatttta	1620
tctaaatcta cagggcctta taagtgcctc caaactaaat acttagttgg cataggtgag	1680
cactgttctg gtcttgctat taaaagtgat tattgtggag gcaatccttg tacttgccaa	1740
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tttgctaatt ttattttgca tgggtgtaat agtggtagta cttgttctac tgatttacia	1860
aatcaaaca cagacataat tcttgggtgt tgtgttaatt atgatctta tggattaca	1920
ggccaaggta ttttgttgga ggttaatgcg acttattata atagttggca gaacctttta	1980
tatgattota atggtaatct ctatggtttt agggactact taacaaacag aacttttatg	2040
attcgtagtt gctatagcgg tcgtgtttca gcgggcttct actctaactc ttccgaacca	2100
gcattgctat ttcggaatat taaatgcaat tacgttttta ataatactct ttcacgacag	2160
ctgcaacctt ttaactattt tgatagttat cttgggtgtg ttgtcaatgc tgataatagt	2220

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## FIGURE 3 (Page 2 of 2)

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acttctagtt ctgttcaaac atgtgatctc acagtaggta gtggttactg gggggattac 2280
tctacacaaa gacgaagtcg tagaacgatt accactgggt atcggtttac taattttgag 2340
ccattttactg ttaatccagt aaatgatagt ttacaccctg taggtgggtt gtatgaaatt 2400
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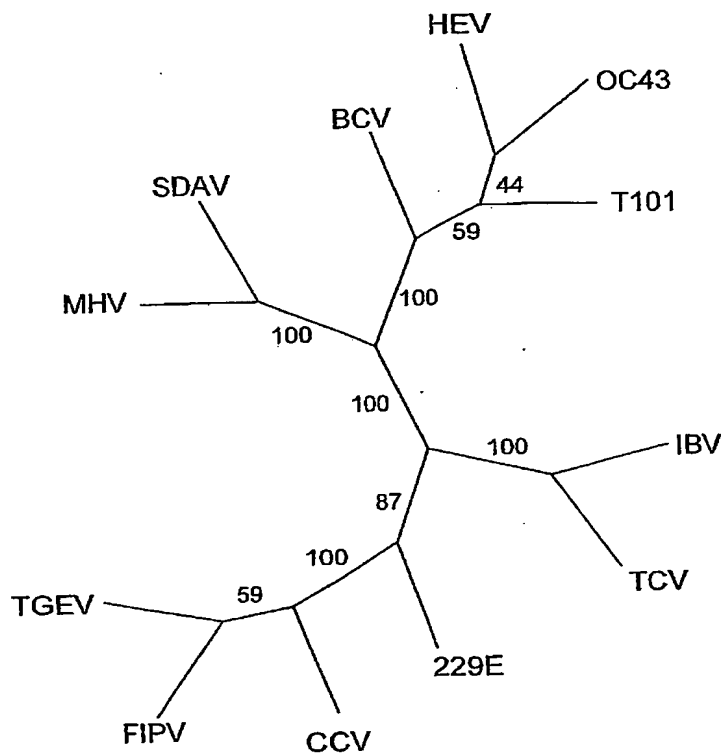
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## FIGURE 4

MFLILLISLP	MAFAVIGDLK	CTTVSINDVD	TGAPSISTDV	VDVTNGLGTY	YVLDRVYLNT	60
TLLNGYYPT	SGSTYRNMAL	KGTLLLSTLW	EKPPFLSDFI	DGVFAKVNT	KVIKDGVVYS	120
EFFAITIGST	FVNTSYSVVV	QPHTTNLDNK	LQGLLEISVC	QYTMCDYPET	MCHPNLGNKR	180
IELWHWDTG	VPCLYKRNET	YDVNADYLYS	HFYQEGGTFY	AYFTDTGVVT	KFLFHVYLG	240
VLSHYVMPL	TCNSAMTLEY	WVTPLTFKQY	LLAFNQDQVI	FNAVDCXSDF	MSEIKCKTSL	300
IAPSTGVYEL	NGYTVQPIAD	VYRRIPNLPD	CNIEAWLNDK	SVPSPLNWER	KTFSNCFNM	360
SSLSMFIQAD	SFTCNNIDAA	KIYGMCFESI	TIDKFAIPNG	RKVDLQMGNL	GYLQSFNYRI	420
DTTATSCQLY	YNLPASNVSI	SRFNPSIWNR	RFGFTEQSVF	KPQPVGVFTD	HDVVYAQHCF	480
KAPTNCPCCK	LNGSLCVGSG	FGIDAGYKNS	GIGTCFAGTN	YLTCTYANQC	DCLCTPDPII	540
SKSTGPYKCP	QTKYLVGIGE	HCSGLAIKSD	YCGGNPCTCQ	PKAFLGWSVD	SCLQGDRCNI	600
FANFILHGVN	SGTTCSTDLO	KSNTDIILGV	CVNYDLYGIT	GQGIFVEVNA	TYYNWQNL	660
YDSNGNLYGF	RDYLTNRFTM	IRSCYSGRVS	AGFHSNSSEP	ALLFRNIKCN	YVFNNTLRQ	720
LQPINYFDSY	LGCVVNADNS	TSSSVQTCDL	TVGSGYWGDI	STQRRSRRTI	TTGYRFTNFE	780
PFTVNPVND	LHPVGGLYEI	QIPSEFTIGN	MEEFIQTRSP	KVTIDCPVFW	CGDYAACKSQ	840
LVEYGSFCDN	INAILTEVNE	LLDTTOLQVA	NSLMNGVTLS	TKLKGDFNFN	VDDINFSPVL	900
GCLGSECNKV	SSRSAIEDLL	FSKVKLSDVG	FVDAYNNCTG	GAEIRDLCIV	QSYNGIKVLP	960
PLLSNQISG	YTLAATFASL	FPPWSAAAGV	PFFLVNQYRI	NGIGVTMDVL	TQNKQLISNA	1020
FNNALDAIQE	GFDATNSALV	KIQAVVNANA	EALNNLLQQL	SNKFGAISAS	LQEILSRLDA	1080
LEAQAQIDRL	INGRLTALNA	YVSQQLSDST	LVKFSAQAAM	EKVNECVKSO	SSRINFCGNG	1140
NHIISLVQNA	PYGLYFIHFS	YVPTKYVTAK	VSPGLCIAGD	RGIAPKSGYF	VNVNNTWMFT	1200
GSYYYYPEPI	TGNNVVVMST	CAVNYTKAPD	VMLNISTPNL	PDFKEELDQW	FKNQTLMAPD	1260
LSLDYINVTF	LDLQDEMNR	QEAIVLNHS	YINLKDIGTY	EYYVKWPWYV	WLLIGLAGVA	1320
MLVLLFFICC	CTGCSTSCFK	KCGGCCDDYT	GHQELVIKTS	HDD		1363

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**FIGURE 5**



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## FIGURE 6

T101 CTCAGATGAATTTGAAATATGCTATTAGTGCTAAGAATAGAGCCCGCACTGTTGCTGGTG  
BCV CTCAAATGAATTTGAAATATGCTATTAGTGCTAAGAATAGAGCCCGCACTGTTGCTGGTG  
OC43 CTCAAATGAATTTGAAATATGCTATTAGTGCTAAGAATAGAGCCCGCACTGTTGCTGGTG  
HEV CTCAAATGAATTTGAAATATGCTATTAGTGCCAAGAATAGAGCCCGCACTGTTGCTGGTG  
CCV CTCAGATGAATTTGAAATATGCTATTTCTGGAAAGGCTAGAGCTCGTACAGTAGGAGGAG  
\*\*\*\*\* \*\* \*\*\* \*\*\*\*\* \*\* \* \* \* \* \*

T101 TTTCCATACTTAGTACTATGACTGGCAGAATGTTTCATCAAAAATGTTTGAAAAGTATAG  
BCV TTTCCATACTCAGTACTATGACTGGCAGAATGTTTCATCAAAAATGTTTGAAAAGTATAG  
OC43 TTTCCATACTTAGTACTATGACTGGCAGAATGTTTCATCAAAAATGTTTGAAAAGTATAG  
HEV TTTCCATACTTAGTACTATGACTGGCAGAATGTTTCATCAAAAATGCTTGAAAAGTATAG  
CCV TTTCACTTCTTTCTACCATGACTACGAGACAATACCACCAGAAGCATTTGAAGTCAATTG  
\*\*\*\*\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

T101 CAGCTACACGTGGTGTTCCCTGTTGTTATAGGCACCACTAAATTTTATGGCGGCTGGGATG  
BCV CAGCTACACGTGGTGTTCCCTGTTGTTATAGGCACCACTAAGTTTATGGCGGCTGGGATG  
OC43 CAGCTACACGTGGTGTTCCCTGTAGTTATAGGCACCACTAAATTTTATGGCGGCTGGGATG  
HEV CAGCTACACGTGGCGTTCCCTGTGTTATAGGCACCACTAAATTTTATGGCGGCTGGGATG  
CCV CTGCAACACGCAATGCCACTGTGGTTATTGGCTCAACCAAGTTTATGGTGGTTGGGATA  
\* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

T101 ATATGTTACGTCGCCCTTATTAAAGATGTTGACAATCCTGTACTTATGGGTTGGGATTATC  
BCV ATATGTTACGTCGCCCTTATTAAAGATGTTGATAATCCTGTACTTATGGGTTGGGATTATC  
OC43 ATATGTTACGCCGCCCTTATTAAAGATGTTGACAATCCTGTACTTATGGGTTGGGATTATC  
HEV ATATGTTACGCCGCCCTTATTAAAGATGTTGATAATCCTGTACTTATGGGTTGGGATTATC  
CCV ACATGCTTAAAAATTTAATGCGTGATGTTGATAATGGTTGTTTGATGGGATGGGACTATC  
\* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

T101 CTAAGTGTGA  
BCV CTAAGTGTGA  
OC43 CTAAGTGTGA  
HEV CAAAGTGTGA  
CCV CTAAGTGTGA  
\* \* \* \* \* \*

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## FIGURE 7

prothCVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
proHEVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
protBCVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
<b>protCRCVpol</b>	<b>---QNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR</b>
protCECVpol	MTQMNLKYAISGKARARTVGGVSLSTMTTROYHQHCLKSIAATR
	*****.* *****.***:***** * :*** *****
prothCVpoly	GVPVVGTTKFGYGGWDDMLRRLIKDVDPVLMGWDYPKC
proHEVpoly	GVPVVGTTKFGYGGWDDMLRRLIKDVDPVLMGWDYPKC
protBCVpoly	GVPVVGTTKFGYGGWDDMLRRLIKDVDPVLMGWDYPKC
<b>protCRCVpol</b>	<b>GVPVVGTTKFGYGGWDDMLRRLIKDVDPVLMGWDYPKC--</b>
protCECVpol	NATVVGSTKFGYGGWDDNMLKNLMRDVDNGCLMGWDYPKC---
	...*****:*****:***.*:***:***:*** *****

BNSDOCID: <WO 2004011651A1\_1\_>



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FIGURE 8 (Page 2 of 9)

CRCVspike	-TGTGCGATTACCCACATA-CGATGTGTC-ATCCTAATCTGGGT-AATAAACG--CATAG
CECVspike	ATTTATGGTCTTGAGTGGGAATGATGAATTTGTTACAGCGTACATTAGTGGTCGTTCTTAT
	* * * * * * * * * * * * * * *
CRCVspike	AACATATGGCATTGGGATACAGGTGTTGTTCCCTGTT-TATATAAGCGTAATTTACATAT
CECVspike	AATTGGAACATCAATAATAATTGGTTTAAACAATGTCACGCTTCTGTATAGTCGCTCAAGC
	* * * * * * * * * * * * * * *
CRCVspike	GATGTGA-ATGCTGATTATTTGTATTCCCATTTTATCAAGAAGGTGGTACTTT---TTA
CECVspike	ACTGCCACATGGCAACACAGTGC-TGCATACGTTACCAAGGTGTTTCTAACTTCACCTA
	* * * * * * * * * * * * * * *
CRCVspike	TGCATATTTTACAGACACTGGTGTGTTACTAAGTTTCTGTTTCATGTTTAT-TTAGGCA
CECVspike	TTACAAGTTAAATAACACCAATGGTCTAA--AAACCTATGAATTATGTGAAGATTATGAA
	* * * * * * * * * * * * * * *
CRCVspike	CGGTGCTTT---CACATTATTA-TGTCATGCCCTTGACTTGTAATAGTGCTATGACTTTA
CECVspike	TATTGCACTGGCTACGCCACTAACATCTTTGCCCAACTGTGGGAGGTTACATACCTGAT
	* * * * * * * * * * * * * * *
CRCVspike	GAATACTGGGTTA-----CACCTCTCACTTTTAAACAATATTTACTCGCTTTCAATCAAG
CECVspike	GGATTTAGTTTTTAAACAATTGGTTTTTGCTTACAAACAGCTCCACTTTTGTAGTGGCAGA
	* * * * * * * * * * * * * * *
CRCVspike	ATGGTGTTATTTTTAATGCTGTTGATTGTAAGAGTGATTTTATGAGTGAGATTAAGTGT-
CECVspike	TTTGTAACAAATCAACCATTATTAGTTAATTGCTTGTGGCCAGTTCCTAGTTTTGGTGTT
	* * * * * * * * * * * * * * *
CRCVspike	--AAACACTATCTATAGCACCATCTACTGGTGTATGAATTAAACGGTTACACTGTTC
CECVspike	GCAGCACAGAATTTTGTGTTTGAAGGTGCACAGTTTAGCCAATGTAATGGTGTGTTTTTA
	* * * * * * * * * * * * * * *
CRCVspike	AGCCA-ATTGCAGATGTTTACCGACGTATACCTAATCTTCCCG--ATTGTAATATAGAGG
CECVspike	AATAACACAGTAGATGTCATTAGATTCAACCTTAATTTTACTGCAGATGTACAATCTGGC
	* * * * * * * * * * * * * * *

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## FIGURE 8 (Page 3 of 9)

CRCVspike	CTTGGCTTAATGATAAGT-CGGTGCCTTCTCCATTAAATTGGGAACGTAAGACCTTTTCA
CECVspike	ATGGGTGCTACAGTATTTTCACTGAATACAACAGGTGGTTGCATTCTTGAGATTTCTT--
	* * * * *
CRCVspike	AATTGTAATTTTAATATGAGCAGCCTGATGTCTTTTATCCAGGCTGACTCGTTTACTTGT
CECVspike	-GTTATAATGATATAGTGAGCGAGTCAAGTTTCTACAGTTATGGTGA---AATTCCCTTC
	* * * * *
CRCVspike	AATAATATTGATGCTGCTAAGATATACGGTATGTGTTTTTTTCA--GCATAACTATAGATA
CECVspike	GGCGTAACTGATGG-ACCGCGTTAT-TGTTATGTCCTCTATAATGGCACAGCTCTTAAGT
	* * * * *
CRCVspike	AGTTTGCTA---TACCCAATGGTAGGAAGGTTGACCTACAAATGGGCAATTTGGGCTATT
CECVspike	ATTTCCGGCACATTACCCCTAGTGCTCAAGG--AAATTGCTATTAG-TAAGTGGGGCCCAAT
	* * * * *
CRCVspike	TGCAGTCTTTTAACTATAGAATTGATACTACTGCTACAAGTTGTCAGTTGTATTATAATT
CECVspike	TTTATATTAATGGTTACAATTTCTTTAGCACTTTTCTATTGATTGATATCTTTTAACT
	* * * * *
CRCVspike	TACCTGCTAGTAATGTTTCTATTAGCAGGTTTAAATCCTTCTATTGGAATA--GGAGATT
CECVspike	TAACCACTGGTGATAGTGGAGCATTTTGGACAATTGCTTACACATCGTACACTGAAGCAT
	* * * * *
CRCVspike	TGGTTTTA-CAGAACAATCTGTTTTTAAGCCT-CAACCTGTAGGTGTTTTTACTGATCAT
CECVspike	TAGTACAAGTTGAAAACACAGCCATTAAAAGGTGACGTATTGTAACAGTCAC-ATTAAT
	* * * * *
CRCVspike	GATGTTGTTTATGCACAACATTGTTTTAAAGCTCCCACAAATTTCTGTCCG-----TGTA
CECVspike	AACATCAAATGTTCTCAACTTACTGCTAATTTGCAAAATGGCTTTTATCCTGTTGCTTCA
	* * * * *
CRCVspike	AATTGAATGGGTCTTTGTGTGTAGGTAGTGGTTTTGGTA--TAGATGCTGGTTATAAA--
CECVspike	AGTGAAGTTGGTCTTGTCAATAAGAGTGTGTGTACTACCTAGTTTCTATTACATACC
	* * * * *

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FIGURE 8 (Page 5 of 9)

CRCVspike	ATGATTCGTAGTTGCTATAGCG-GTCGTGTTTCAGCGGGCTTTCA---CTCTAACTCTTC
CECVspike	ATACTTAGTGGCTTACATTATACATCACTATCAGGTGATTATTAGGTTTTAAAAATGTT
	**   ***   *   *   *   *   *   *   *   *   *   *   *   *
CRCVspike	CGAACCAGCATTG-CTATTTTCGGAATATTAAATGCAATTACGTTTTTAATAATACTCTTT
CECVspike	AGTGATGGTGTGTCTATTCTGTGACACCATGTGATGTAAGCGCACAAGCGGCTGTTATT
	*       *   ***   *****   *   *   *   *   *   *       *       *   **
CRCVspike	CACG-----ACAGCTGCAACCTATTAACATTTTGATAGTTATCTTG GTTGTGTTGTCAA
CECVspike	GATGGGGCCATAGTTGGAGC-TATGACTTCCATTAATAGTGAAC-TTTAGGTCTAACAC
	*   *       *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CRCVspike	TGCTGATAATAGTAC-----TTCTAGTTCTGTTCAAACATGTGATCTCACAGTAGGTAGT
CECVspike	ATTGGACAACAACACCAAATTTTTATTACTACTCTA-TATATAAT---ACAACAAATGAG
	*   *   *   *   *       *   *   *   *   *   *   *   *   *   *   *   *
CRCVspike	GGTTACTGGGGGGATTACTCTACACAAAGACGAAGT-----CGTAGAACGATTACCACTGG
CECVspike	AGA-ACTCGTGGCACTGCAATCGACAGTAACGATGTAGATTGTGAACCTATCATAACCTA
	*   ***   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CRCVspike	TT-----ATCGGTTT-----ACTAATTTTGAGCCATTTACTGTTAATCCAGTAAATGATAG
CECVspike	TTCTAACATAGGTGTTTGTAAAAATGGTGCGTTGGTTTTTTATTAACGTCACACATTCTGA
	**       *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CRCVspike	TTTACACCCTGTAGGTGGTTGTAT--GAAAT-TCA-AATACCTTCAGAGTTTACTATAG
CECVspike	TGGAGATGTT-CAACCAATTAGCACTGGCAATGTCACGATACCCACAACTTTACCATAT
	*   *   *   *   *       *   *   *   *   *   *   *   *   *   *   *
CRCVspike	GTAATATGGAGGAGTTTATTCAAACAAGATCTCCTAAAGTTACTATTGATTGTCCTGTTT
CECVspike	CTGTGCAAGTTGAATACATCCAGGTTTACACTACACCGGTGTCAATAGATTGTTCTAGAT
	*       *   *   *   *   *       *   *   *   *   *   *   *   *   *
CRCVspike	TTGTCTGTGGTGATTATGCAGCATGTAAATCACAGTTGGTTGAATATGGTAGTTTTGTG
CECVspike	ACGTTTGTAATGGTAACCCTAGATGTAATAAATTGTTAACACAATATGTTTCTGCATGTC
	**   ***   *   *   *   *   *   *   *   *   *   *   *   *   *   *

## FIGURE 8 (Page 6 of 9)

```
CRCVspike   ACAATATTAATGCTATACTCACAG-AAGT-----AAATGAACTACTTGACACTA
CECVspike   AAACATTTGAGCAAGCGCTTGCAATGAGTGCCAGCCTTGAAAACATGGAAGTTGATTCCA
* * * * *      * *      * *      * *      * *      * *      * *
* * * * *      * *      * *      * *      * *      * *      * *

CRCVspike   CACAGTTGCAAGTAGCTAATAGTTTAAATGAATGGTGTCACTCTTAGCACTAAGCTTAAAG
CECVspike   TGTGTGTTGTTTCAGAAAATGCCCTTA-AATTGGCATCTGTTGAGGCGTTCAATAGTACA
      * * *      * *      * *      * *      * *      * *      * *
      * *      * *      * *      * *      * *      * *      * *

CRCVspike   ATGGCTTTAATTTCAATGTAGATGACAT----CAATTT---TTCCCCTGTATTAGGTTGT
CECVspike   GAACATTTAGATCCTATTACAAAGAATGGCCTAACATAGGTGGTTCCTGGCTAGGAGGT
      * * *      * *      * *      * *      * *      * *      * *
      * *      * *      * *      * *      * *      * *      * *

CRCVspike   TTAGGAAGCGAAT-----GTAATAA-AGTTTCCAGTA--GATCTGCTATAGAGGAT
CECVspike   CTAAAAGACATACTTCCGTCCCATAGCAACGTAAGTATCGTTCTGCTATAGAAGAC
      * *      * *      * *      * *      * *      * *      * *
      * *      * *      * *      * *      * *      * *      * *

CRCVspike   TTACTTTTTTCTAAAGTAAAGTTATCTGATGTTGGTTTGTGTTGATGC---TTATAATAAT
CECVspike   TTGCTTTTTGATAAAGTTGTAACCTCTGGTCTAGGTACAGTTGATGAAGATTATAAACGT
* * * * *      * * * *      * * * *      * * * *      * * * *      * *
* * * * *      * * * *      * * * *      * * * *      * * * *      * *

CRCVspike   TGTACTGGAGGTGCCGAAATTAGGGACCTCATTTGTGTGCAAAGTTATAATGGTATCAAA
CECVspike   TGTACAGGTGGTTATGACATAGCTGACTTAGTTTGTGCACAATATTACAATGGCATCATG
* * * * *      * * * *      * * * *      * * * *      * * * *      * *
* * * * *      * * * *      * * * *      * * * *      * * * *      * *

CRCVspike   GTGTTGCCTC-CACTGCTCTCAGAAAATCAGATCAGTGGATACACTTTGGCTGCCACCTT
CECVspike   GTTCTACCTGGTGTGCTAAT-GATGACAAGATGACTATGTACACAGCCTCTCTTGCAGG
* *      * * *      * * *      * *      * *      * *      * *      * *
* *      * * *      * * *      * *      * *      * *      * *      * *

CRCVspike   TGCTAGTCTGTTTCCTCC-TTGGTCAGCAGCA--GCAGGCGTACCATTTTATTTAAATGT
CECVspike   TGGTATAGCATTAGGTGCACTAGGTGGTGGCGCCGTGGCTATACCTTTTGCAGTAGCAGT
* *      * *      * *      * *      * *      * *      * *      * *
* *      * *      * *      * *      * *      * *      * *      * *

CRCVspike   TCAGTATCGTATTAATGGTATTGGTGTACCATGGATGTGCTAACTCAAATCAAAGCT
CECVspike   TCAGGCTAGACTTAATTATGTGCTCTACAACTGATGTATTGAACAAAAACCAGCAGAT
* * * *      * *      * * * *      * * * *      * *      * *      * *
* * * *      * *      * * * *      * * * *      * *      * *      * *
```

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## FIGURE 8 (Page 7 of 9)

```
CRCVspike      TATTTCTAATGCATTTAACAATGCCCTTGATGCTATT-----CAGGAAGGGTT--
CECVspike      CCTGGCTAATGCTTTCAACCAAGCTATTGGTAACATTACACAGGCATTGGTAAGGTTAA
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TGATGCTA-----CCAATTCTGCT-----TTAGTTAAAT
CECVspike      TGATGCTATACATCAAACATCACAAGGTCTTGCCACTGTTGCTAAAGCATTGGCAAAAGT
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TCAAGCTGTTGTTAATGCAAATGCTGAAGCTCTTAATAACTTATTGCAACAACTCTCTAA
CECVspike      GCAAGATGTTGTTAACACACAAGGGCAAGCTTTAAGCCACCTAACAGTACAAGTCAAA
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TAAATTTGGTGCTATAAGTGCTTCTTTACAAGAAATTCTATCTAGACTTGATGCTCTTGA
CECVspike      TAGCTTCCAAGCCATTAGTAGTCTTATTAGTGACATTTATAATAGGCTTGATGAAGTGA
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      AGCGCAAGCTCAGATAGACAGACTTATCAATGGGCGTCTTACCGCTCTTAATGCTTATGT
CECVspike      TGCTGATGCACAAGTTGATAGGCTGATTACAGGTAGACTTACAGCACTTAATGCATTTGT
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TTCTCAACAGCTTAGTGATTCTACACTAGTAAAATTTAGTGACAGCACAAGCTATGGAGAA
CECVspike      ATCTCAGACTCTAACCAGACAAGCGGAGGTTAGGGCTAGTAGACAAGTGGCAAGACAA
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      GGTAAATGAATGTGTCAAAGCCAATCATCTAGGATAAATTTTGTGGTAATGGTAATCA
CECVspike      GGTAAATGAATGTGTTAGGTCTCAGTCTCAGAGATTTGGATTTTGTGGTAATGGTACACA
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TATTATATCATTAGTGAGAATGCTCCATATGGTTTGTATTTATCCACTTTA-GCTATG
CECVspike      TTTGTTTTCACTTGCAAATGCAGCACCAATGGCATGGTTTTCTTTCACACAGTGCTAT-
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

CRCVspike      TCCCTACTAAGTATGTCAGTGCAGAGGTTAGTCCCGGTCTGTGCATYGCAGGTGATAGAG
CECVspike      TACCAACAGCTTATGAACTGTAACAGCTTGGTCAGGTATTTGTGCTTCAGATGGCGATC
                *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
```

## FIGURE 8 (Page 8 of 9)

CRCVspike      GTA-----TAGCTCCTAAGAGTGGTTATTT-----TGTT----AATGTAAATAACA  
CECVspike      GCACTTTTGGACTTGTCTGTTAAAGATGTTTCAGTTGACGTTGTTTCGTAATCTAGATGACA  
                 \* \*               \* \*               \* \* \*               \* \* \* \* \*               \* \* \* \*               \* \* \* \* \* \* \*

CRCVspike      CTTGGATGTTCACTGGTAGTGGTTATTACTACCTGAACCTATAACTGGAAATAATGTGG  
CECVspike      AGTTCTATTTGACTCCCAGAACTATGTATCAGCCTAGAGCTGCAACTAGTTCTGATTTTG  
                 \*               \* \* \* \*               \* \*               \* \*               \* \* \* \*               \* \*               \* \* \* \* \*               \* \* \* \* \*

CRCVspike      TTGTTATGAGTACCTGTGCTGTTAACTATACTAAAGCACCAGGATGTAATGCTGAACATTT  
CECVspike      TTCAGATTGAGGGGTGCGACGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCTTAGTA  
                 \* \*               \* \*               \* \* \*               \* \*               \* \*               \* \* \* \* \*               \*               \* \*               \* \*

CRCVspike      CAACACCCAACCTCCCTGATTTTAAGGAAG-----AGTTGCATCAATGTTTAAAAAC  
CECVspike      TTATACCTGACTATATCGACATTAATCAGACTGTTCAAGACATATTAGAAAACACAGAC  
                 \* \* \* \*               \* \*               \* \* \* \*               \*               \* \*               \* \*               \* \* \* \* \*

CRCVspike      CAAACATTAATGGCACCAGATTTGTCACCTGATTATATAAATGTTACATTCTTGGACCTA  
CECVspike      CAAAC-TGGACTGTACCTGAATTGACAATTGACATTTTTAACGCAACCTATTTAAATCTG  
                 \* \* \* \*               \*               \* \* \* \*               \* \* \* \*               \* \* \* \*               \* \* \* \*               \* \* \* \* \*

CRCVspike      CAAGATGAAATGAATAGGTTACAGG--AGGCAATAAAAGTT---TTAAATCATAGC----  
CECVspike      ACTGGTGAAATTGATGACTTAGAATTTAGGTCAGAAAAGCTACATAACACCACAGTAGAG  
                 \*               \* \* \* \*               \* \*               \* \*               \* \* \* \*               \*               \* \* \* \* \*

CRCVspike      -----TACAT---CAATCTCAAGGACATTGGTACA  
CECVspike      CTTGCCATTCTCATTGACAATATTAACAATACATTAGTCAATCTTGAATGGCTCAATAGA  
                 \* \* \* \*               \* \* \* \*               \*               \*               \* \* \*

CRCVspike      TATGAATATTATGTAAATGGCCTTGGTATGTATGGCTTTTAATTGGCCTTGCTGGCGTA  
CECVspike      ATTGAACTTATGTGAAATGGCCTTGGTATGTGTGGCTACTAATAGGC-TTAGTAGTAGT  
                 \* \* \* \*               \* \* \* \*               \* \* \* \*               \* \* \* \*               \* \* \* \*               \* \* \* \* \*

CRCVspike      GCTATGCTTGTT-TTACTATTCTTCATATGCTGTTGTACAGGATG--TGGGACTAGTTG  
CECVspike      GTTTTGCATACCGCTATTGCTATTTTGTCTGTTGTAGTACAGGTTGCTGTGGATGCATAGG  
                 \* \* \* \*               \* \*               \* \*               \* \* \* \*               \* \* \* \*               \* \*               \* \* \*

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## FIGURE 8 (Page 9 of 9)

```
CRCVspike      TTTTAAGAAATGCGGTGGTTGTTGTGATGATTATACTGGACA--TCAGGAGTTAGTAATC
CECVspike      TTGTTTGGGAAGTTGTTGTCATTCTATTTGTAGTAGAAGACAATTGAAAATTACGAACC
                ** * * * * ** * * * * * * * * * * * * * * * * * *
                *

CRCVspike      AA----AACGTCACATGACGACTAA-----
CECVspike      AATTGAAAAAGTGCATGTCCACTAAA-----
                **      **      ***** * *****
```



## FIGURE 9 (Page 1 of 12)

```
BCVspike      ATGTTTTTGATACTTTTAATTCCTTACCAATGGCTCTTGCTGTTATAG
HCVspike      -----ATGTTTTTGATACTTTTAATTCCTTACCAACGGCTTTTGCTGTTATAG
CRCVspike     -----ATGTTTTTGATACTTTTAATTCCTTACCAATGGCTTTTGCTGTTATAG
HEVspike      -----ATGTTTTTTATACTTTTAATCACCCCTGCCTTCGTGTTTTGCAGTTATAG
               *****  *****  ** * **  * * ****  *****

BCVspike      GAGATTTAAAGTGTA TACTACGGTTTCCATTAATGATGTTGACACCGGTGTTCCCTTCTGTTA
HCVspike      GAGATTTAAAGTGTA TACTACGGTTTCCATTAATGATATTGACACCGGTGCTCCTTCTATTA
CRCVspike     GAGATTTAAAGTGTA TACTACGGTTTCCATCAATGATGTTGACACCGGTGCTCCTTCTATTA
HEVspike      GGGATTTAAAGTGTAATACTTCATCAATTAATGACGTTGACACTGGTGTGCCATCTATTA
               * *****  ***  ** ** *****  *****  *****  ** *** **

BCVspike      GCACTGATACTGTCGATGTTACTAATGGTTTAGGTACTTATTATGTTTTAGATCGTGTGT
HCVspike      GCACTGATATTGTCGATGTTACTAATGGTTTAGGTACTTATTATGTTTTAGATCGTGTGT
CRCVspike     GCACTGATGTTGTCGATGTTACTAATGGTTTAGGTACTTATTATGTTTTAGATCGTGTGT
HEVspike      GCTCTGAAGTTGTTGATGTCACTAATGGTTTGGGGACTTCTATGTTTTAGATCGTGTCT
               ** *****  *** *****  *****  ** *****  *****  *****

BCVspike      ATTTAAATACTACGTTGTGCTTAATGGTTACTACCCTACTTCAGGTTCTACATATCGTA
HCVspike      ATTTAAATACTACGTTGTGCTTAATGGTTACTACCCTACTTCAGGTTCTACATATCGTA
CRCVspike     ATTTAAATACTACATTGTTGCTTAATGGTTATTATCCTACTTCAGGTTCTACATATCGTA
HEVspike      ATTTAAATACCACATTGTTGCTCAATGGTTATTACCCAATTCAGGTGCTACATTTCGTA
               *****  ** *****  *****  ** * * *****  *****  *****

BCVspike      ATATGGCACTGAAGGGAAC TTTACTATTGAGCACACTATGGTTTAAACCACCTTTTCTTT
HCVspike      ATATGGCACTGAAGGGAAC TTTACTATTGAGCAGACTATGGTTTAAACCACCTTTTCTTT
CRCVspike     ATATGGCACTGAAGGGAAC TTTACTATTGAGCACACTATGGTTTAAACCACCTTTTCTTT
HEVspike      ATGTGGCTCTGAAAGGAAC TCGATTATTGAGCACCTTGTGGTTTAAAGCCGCCTTTTCTTT
               ** **** *****  *****  * *****  * *****  ** * * *

BCVspike      CTGATTTTATTAATGGTATTTT TGCTAAGGTCAAAAATACCAAGGTTATTAAAAATGGTG
HCVspike      CTGATTTTATTAATGGTATTTT TGCTAAGGTCAAAAATACCAAGGTTATTAAAAAGGGTG
CRCVspike     CTGATTTTATTGATGGTGT TTTTGCTAAGGTAAAAAATACCAAGGTTATTAAAGATGGTG
HEVspike      CACCTTTTAAATGATGGTATTTT TGCCAAGGTTAAAAACAGCAGATTTCTAAACATGGTG
               * *****  * *****  *****  *****  *****  * **  ** *****  *
```

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## FIGURE 9 (Page 2 of 12)

```
BCVspike    TAATGTATAGTGAGTTTCCTGCTATAACTATAGGTAGTACTTTTGTAATACATCCTATA
HCVspike    TAATGTATAGTGAGTTTCCTGCTATAACTATAGGTAGTACTTTTGTAATACATCCTATA
CRCVspike   TAGTGTATAGTGAGTTTCCTGCTATAACTATAGGTAGTACTTTTGTAATACATCCTATA
HEVspike    TTATTTATAGTGAGTTTCCTGCTATTACTATAGGTAGTACTTTTGTAATACTTCCTATA
*  *  *****

BCVspike    GTGTGGTAGTACAACCACATACTACCAATTTAGATAATAAATTACAAGGTCTCTTAGAGA
HCVspike    GTGTGGTAGTACAACCACATACTACCAATTTGGATAATAAATTACAAGGTCTCTTAGAGA
CRCVspike   GTGTGGTAGTACAACCACATACTACTAATTTAGATAATAAATTACAAGGTCTCTTAGAGA
HEVspike    GCATAGTAGTAAAGCCTCATACCTCATTATTAATGGTAATTTACAAGGTTTTTGCAAA
*  *  *****

BCVspike    TCTCTGTTTGCCAGTATACTATGTGCGAGTACCCACATACGATTTGTCATCCTAATTTGG
HCVspike    TCTCTGTTTGCCAGTATACTATGTGCGAGTACCCACATACGATTTGTCATCCTAATCTGG
CRCVspike   TCTCTGTTTGCCAGTATACTATGTGCGAGTACCCACATACGATGTGTCTATCCTAATCTGG
HEVspike    TTTCTGTTTGTCAATATACTATGTGTGAATACCCACAGACTATTTGTCATCCTAATTTGG
*  *****

BCVspike    GTAATCGGCGCATAGAACTATGGCATTGGGATACAGGTGTTGTTTCCTGTTTATATAAGC
HCVspike    GTAATCGACGCGTAGAACTATGGCATTGGGATACAGGTGTTGTTTCCTGTTTATATAAGC
CRCVspike   GTAATAAACGCATAGAACTATGGCATTGGGATACAGGTGTTGTTCCCTGTTTATATAAGC
HEVspike    GTAATCAACGCATAGAACTATGGCATCATGACACAGATGTTGTTTCCTGTTTATACAGGC
*****

BCVspike    GTAATTTACATATGATGTGAATGCTGATTATTTGTATTCCATTTTATCAAGAAGGTG
HCVspike    GTAATTTACATATGATGTGAATGCTGATTACTTGTATTCCATTTTATCAAGAAGGTG
CRCVspike   GTAATTTACATATGATGTGAATGCTGATTATTTGTATCCATTTTATCAAGAAGGTG
HEVspike    GTAATTTACATATGATGTGAATGCTGATTATTTATATTTTCACTTTTATCAGGAAGGTG
*****

BCVspike    GTACTTTTTATGCATATTTTACAGACACTGGTGTTGTTACTAAGTTTCTGTTTAAATGTTT
HCVspike    GTACTTTTTATGCATATTTTACAGACACTGGTGTTGTTACTAAGTTTCTGTTTAAATGTTT
CRCVspike   GTACTTTTTATGCATATTTTACAGACACTGGTGTTGTTACTAAGTTTCTGTTTCATGTTT
HEVspike    GCACTTTTTATGCATACTTTACAGATACTGGTTTTGTGACCAAGTTTCTGTTTAAAGTTGT
*  *****
```

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## FIGURE 9 (Page 3 of 12)

BCVspike ATTTAGGCACGGTGCTTTCACATTATTATGTCATGCCCTTGACTTGTAATAGTGCTATGA  
HCVspike ATTTAGGCACGGTGCTTTCACATTATTATGTCCTGCCCTTGACTTGTAATAGTGCTATGA  
CRCVspike ATTTAGGCACGGTGCTTTCACATTATTATGTCATGCCCTTGACTTGTAATAGTGCTATGA  
HEVspike ATTTAGGCACTGTGCTGTCACACTATTATGTTATGCCATTGACTTGTGATAGCGCTTTAT  
\*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

BCVspike CTTTAGAATATTGGGTTACACCTCTCACTTCTAAACAATATTTACTCGCTTTCAATCAAG  
HCVspike CTTTAGAATATTGGGTTACACCTCTCACTTCTAAACAATATTTACTAGCTTTCAATCAAG  
CRCVspike CTTTAGAATACTGGGTTACACCTCTCACTTTTAAACAATATTTACTCGCTTTCAATCAAG  
HEVspike CTTTAGAATATTGGGTTACACCTCTCACTACTAGACAATTTCTTCTAGCCTTTGACCAGG  
\*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

BCVspike ATGGTGTTATTTTAAATGCTGTTGATTGTAAGAGTGATTTTATGAGTGAGATTAAAGTGTA  
HCVspike ATGGTGTTATTTTAAATGCTGTTGATTGTAAGAGTGATTTTATGAGTGAGATTAAAGTGTA  
CRCVspike ATGGTGTTATTTTAAATGCTGTTGATTGTAAGAGTGATTTTATGAGTGAGATTAAAGTGTA  
HEVspike ATGGTGTTTATACCATGCTGTTGATTGTGCTAGTGATTTTATGAGTGAGATTATGTGTA  
\*\*\*\*\* \* \* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

BCVspike AAACACTATCTATAGCACCATCTACTGGTGTTTATGAATTAAACGGTTACACTGTTTCAGC  
HCVspike AAACACTATCTATAGCACCATCTACTGGTGTTTATGAATTAAACGGTTACACTGTTTCAGC  
CRCVspike AAACACTATCTATAGCACCATCTACTGGTGTTTATGAATTAAACGGTTACACTGTTTCAGC  
HEVspike AAACACTATCTATAGCACCATCTACTGGTGTTTATGAATTAAACGGTTACACTGTTTCAGC  
\*\*\*\*\* \*\* \* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

BCVspike CAATTGCAGATGTTTACCGACGTATACCTAATCTTCCCGATTGTAATATAGAGGCTTGGC  
HCVspike CAATTGCAGATGTTTACCGACGTATACCTAATCTTCCCGATTGTAATATAGAGGCTTGGC  
CRCVspike CAATTGCAGATGTTTACCGACGTATACCTAATCTTCCCGATTGTAATATAGAGGCTTGGC  
HEVspike CTGTTGCCACTGTGTATCGTAGAATACCTGACTTACCCAATTGCGATATCGAAGCTTGGC  
\* \*\*\*\* \*

BCVspike TTAATGATAAGTCTGTGCCCTCTCCATTAAATTGGGAACGTAAGACCTTTTCAAATTGTA  
HCVspike TTAATGATAAGTCGGTGCCCTCTCCATTAAATTGGGAACGTAAGACCTTTTCAAATTGTA  
CRCVspike TTAATGATAAGTCGGTGCCCTCTCCATTAAATTGGGAACGTAAGACCTTTTCAAATTGTA  
HEVspike TTAATCTAAGACCGTTTCTTTCGCCCTCTAATTGGGAACGTAAGACCTTTTCAAATTGTA  
\*\*\*\*\* \*\*\*\*\* \*

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## FIGURE 9 (Page 4 of 12)

```
BCVspike      ATTTTAATATGAGCAGCCTGATGTCTTTTATTCAGGCAGACTCATTTACTTGTAATAATA
HCVspike      ATTTTAATATGAGCAGCCTGATGTCTTTTATTCAGGCAGACTCATTTACTTGTAATAATA
CRCVspike     ATTTTAATATGAGCAGCCTGATGTCTTTTATCCAGGCTGACTCGTTTACTTGTAATAATA
HEVspike      ATTTTAACATGGGCAGGCTGATGTCTTTTATTCAGGCTGACTCTTTTGGTTGTAACAATA
*****  ***  *****  *****  *****  *****  *****  *****

BCVspike      TTGATGCAGCTAAGATATATGGTATGTGTTTTCCAGCATAACTATAGATAAGTTTGCTA
HCVspike      TTGATGCTGCTAAGATATATGGTATGTGTTTTCCAGCATAACTATAGATAAGTTTGCTA
CRCVspike     TTGATGCTGCTAAGATATACGGTATGTGTTTTCCAGCATAACTATAGATAAGTTTGCTA
HEVspike      TTGATGCTTCTCGCTTATATGGTATGTGTTTTGGTAGCATTACTATTGACAAGTTTGCTA
*****  **  *****  *****  *****  *****  *****

BCVspike      TACCCAATGGTAGGAAGGTTGACCTACAATGGGCAATTGGGCTATTTGCAGTCTTTTA
HCVspike      TACCCAATGGTAGGAAGGTTGACCTACAATGGGCAATTGGGCTATTTGCAGTCTTTTA
CRCVspike     TACCCAATGGTAGGAAGGTTGACCTACAAATGGGCAATTGGGCTATTTGCAGTCTTTTA
HEVspike      TACCCAATAGTAGAAAGGTTGATCTGCAAGTGGGTAAATCTGGTTATTTACAATCTTTTA
*****  *****  *****  **  ***  *****  **  *  **  *****  **  *****

BCVspike      ACTATAGAATTGATACTACTGCTACAAGTTGTCAGTTGTATTATAATTTACCTGCTGCTA
HCVspike      ACTATAGAATTGATACTACTGCTACAAGTTGTCAGTTGTATTATAATTTACCTGCTGCTA
CRCVspike     ACTATAGAATTGATACTACTGCTACAAGTTGTCAGTTGTATTATAATTTACCTGCTAGTA
HEVspike      ATTATAAGATTGACACTGCTGTTAGCAGTTGTCAACTCTATTATAGTTTGCCTGCAGCAA
*  ****  *****  ***  ***  **  *****  *  *****  ***  *****  *

BCVspike      ATGTTTCTGTTAGCAGGTTTAATCCTTCTACTTGAATAGGAGATTGGGTTTACAGAAC
HCVspike      ATGTTTCTGTTAGCAGGTTTAATCCTTCTACTTGAATAGGAGATTGGGTTTACAGAAC
CRCVspike     ATGTTTCTATTAGCAGGTTTAATCCTTCTATTGGAATAGGAGATTGGGTTTACAGAAC
HEVspike      ACGTATCTGTCACTCATTATAATCCTTCATCTTGAACAGAAGGTATGGGTTTAT----T
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

BCVspike      AATCTGTTTTTAAGCCTCAACCTGTAGGTGTTTTTACTGATCATGATGTTGTTTATGCAC
HCVspike      AATCTGTTTTTAAGCCTCAACCTGTAGGTGTTTTTACTCATCATGATGTTGTTTATGCAC
CRCVspike     AATCTGTTTTTAAGCCTCAACCTGTAGGTGTTTTTACTGATCATGATGTTGTTTATGCAC
HEVspike      AATCAGAGTTTGGTTCCAG-----AGGC-CTT-----CATGATGCTGTATATTCAC
****  *  ***  *  **  ***  **  *****  ***  ***  ****
```

## FIGURE 9 (Page 5 of 12)

BCVspike	AACATTGTTTTAAAGCTCCACAAATTTCTGTCCGTGTAAATTGGATGGGTCTTTGTGTG
HCVspike	AACATTGTTTTAAAGCTCCACAAATTTCTGTCCGTGTAAATTGGATGGGTCTTTGTGTG
CRCVspike	AACATTGTTTTAAAGCTCCACAAATTTCTGTCCGTGTAAATTGAATGGGTCTTTGTGTG
HEVspike	AGCAATGTTTTAATACACCTAATACATATTGTCCTTGTA---GAACAAGTC--AATGCA
	* * * * *
BCVspike	TAGGTAGTGGTTCTGGTATAGATGCTGGTTATAAAAATAGTGGTATAGGCACCTGTCTCTG
HCVspike	TAGGTAATGGTCCTGGTATAGATGCTGGTTATAAAAATAGTGGTATAGGCACCTGTCTCTG
CRCVspike	TAGGTAGTGGTTTTGGTATAGATGCTGGTTATAAAAATAGTGGTATAGGCACCTGTCTCTG
HEVspike	TAGGTGGTG---CTGGCACAGGAACCTGTCTCTGTAGGCACCACTGTGCGCAAGTGTGTTTG
	***** * * * * *
BCVspike	CAGGTACTAATTATTTAACTTGTCTATAATGCTGCCCAATGTAATTGTTTGTGCACTCCAG
HCVspike	CAGGTACTAATTATTTAACTTGTCCATAATGCTGCCCAATGTGATTGTTTGTGCACTCCCG
CRCVspike	CAGGTACTAATTATTTAACTTGTCTATAATGCTAACCAATGTGATTGTTTGTGCACTCCAG
HEVspike	CTG---C-AGTTAC--A-----AACGCTACTAAGTGTACTTGTCTGGTGTCAACCAG
	* * * * *
BCVspike	ACCCCATTTACATCTAAATCTACAGGGCCTTATAAGTGCCCCCAAATAAATATTTAGTTG
HCVspike	ACCCCATTTACATCTAAATCTACAGGGCCTTACAAGTGCCCCCAAATAAATACTTAGTTG
CRCVspike	ACCCTATTTTATCTAAATCTACAGGGCCTTATAAGTGCCCCCAAATAAATACTTAGTTG
HEVspike	ATCCTTCCACATATAAAGGTGTAAATGCCTGGACTTGTCCGCAATCTAAAGTTTCTATAC
	* * * * *
BCVspike	GCATAGGTGAGCACTGTTTCGGGTCTTGCTATTAAAAGTGATTATTGTGGAGGTAATCCTT
HCVspike	GCATAGGTGAGCACTGTTTCGGGTCTTGCTATTAAAAGTGATTATTGTGGAGGTAATCCTT
CRCVspike	GCATAGGTGAGCACTGTTCTGGTCTTGCTATTAAAAGTGATTATTGTGGAGGCAATCCTT
HEVspike	AACCAGGTGAGCATTGCCCTGGCTTGGGTCTTGTTGGAGGATGATTGCTCTGGTAATCCTT
	***** * * * * *
BCVspike	GTACTTGCCAACCACAAGCATTTTTGGGTGGTCTGTTGATTCTTGTGTTACAAGGGGATA
HCVspike	GTACTTGCCAACCACAAGCATTTTTGGGTGGTCTGTTGACTCTTGTGTTACAAGGGGATA
CRCVspike	GTACTTGCCAACCAAGCATTTTTGGGTGGTCTGTGGACTCTTGTGTTACAAGGGGATA
HEVspike	GCATTGTAAACCACAGGCTTTCATAGGCTGGAGTTCAGAAACTTGTGTTGCAAAATGGTA
	* * * * *

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## FIGURE 9 (Page 6 of 12)

```
BCVspike      GGTGTAATATCTTTGCTAATTTTATTTTGCATGATGTTAATAGTGGTACTACTTGTTCCTA
HCVspike      GGTGTAATATTTTGGCTAATTTTATTTTGCATGATGTTAATAGTGGTACTACTTGTTCCTA
CRCVspike     GGTGTAATATTTTGGCTAATTTTATTTTGCATGGTGTAAATAGTGGTACTACTTGTTCCTA
HEVspike      GGTGTAATATTTTGGCTAATTTTATTTTGAATGATGTTAATAGCGGTACTACCTGTTCTA
*****

BCVspike      CTGATTTACAAAAATCAAACACAGACATAATTCTTGGTGTGTTGTGTTAATTATGATCTTT
HCVspike      CTGATTTACAAAAATCAAACACAGACATAATTCTTGGTGTGTTGTGTTAATTATGATCTTT
CRCVspike     CTGATTTACAAAAATCAAACACAGACATAATTCTTGGTGTGTTGTGTTAATTATGATCTTT
HEVspike      CTGATTTACAAACAGGGTAATACTAATAATTACTACTGATGTTGTGTTAATTATGACCTAT
*****

BCVspike      ATGGTATTACAGGCCAAGGTATTTTGTGAGGTTAATGCGACTTATTATAATAGTTGGC
HCVspike      ATGGTATTACAGGCCAAGGTATTTTGTGAGGTTAATGCGCCTTATTATAATAGTTGGC
CRCVspike     ATGGTATTACAGGCCAAGGTATTTTGTGAGGTTAATGCGACTTATTATAATAGTTGGC
HEVspike      ATGGCATTACAGGCCAGGGCATACTTATAGAAGTTAATGCCACGTATTATAATAGTTGGC
****

BCVspike      AGAACCTTTTATATGATTCTAATGGTAATCTCTATGGTTTTAGAGACTACTTAACAAACA
HCVspike      AGAACCTTTTATATGATTCTAATGGTAATCTCTATGGTTTTAGAGACTACTTAACAAACA
CRCVspike     AGAACCTTTTATATGATTCTAATGGTAATCTCTATGGTTTTAGGGACTACTTAACAAACA
HEVspike      AGAATCTTCTTTATGATTCTAGTGGTAATCTCTATGGCTTTAGAGATTATTTATCAAATA
****

BCVspike      GAACTTTTATGATTTCGTAGTTGCTATAGCGGTCGTGTTTCAGCGGCCTTTCATGCTAATT
HCVspike      GAACTTTTATGATTTCGTAGTTGCTATAGCGGTCGTGTTTCAGCGGCCTTTCATGCTAACT
CRCVspike     GAACTTTTATGATTTCGTAGTTGCTATAGCGGTCGTGTTTCAGCGGCCTTTCACTCTAACT
HEVspike      GAACCTTTCCTTATTTCGTAGCTGCTATAGTGAAGAGTTTCAGCAGTCTTTCATGCTAACT
****

BCVspike      CTTCCGAACCAGCATTGCTATTTTCGGAATATTAAATGCAATTACGTTTTTAATAATACTC
HCVspike      CTTCCGAACCAGCATTGCTATTTTCGGAATATTAAATGCAATTACGTTTTTAATAATACTC
CRCVspike     CTTCCGAACCAGCATTGCTATTTTCGGAATATTAAATGCAATTACGTTTTTAATAATACTC
HEVspike      CTTCTGAACCAGCTTTGATGTTTCGTAATCTTAAATGCAAGCCACGTTTTTAATTATACCA
****
```

## FIGURE 9 (Page 7 of 12)

BCVspike TTTACGACAGCTGCAACCTATTAACCTATTTTGATAGTTATCTTGGTTGTGTTGTCAATG  
HCVspike TTTACGACAGCTGCAACCTATTAACCTATTTTGATAGTTATCTTGGTTGTGTTGTCAATG  
CRCVspike TTTACGACAGCTGCAACCTATTAACCTATTTTGATAGTTATCTTGGTTGTGTTGTCAATG  
HEVspike TTTTAAGACAAATACAGCTTGTAAATTATTTTGATAGTTACCTTGGTTGTGTTGTAAATG  
\*\*\* \* \*\*\*\* \* \*\* \* \* \*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*

BCVspike CTGATAATAGTACTTCTAGTGCTGTTCAAACATGTGATCTCACAGTAGGTAGTGGTTACT  
HCVspike CTGATAATAGTACTTCTAGTGTTGTTCAAACATGTGATCTCACAGTAGGTAGTGGTTACT  
CRCVspike CTGATAATAGTACTTCTAGTTCTGTTCAAACATGTGATCTCACAGTAGGTAGTGGTTACT  
HEVspike CTTATAATAATACAGCTAGTGCTGTAAGTACTTGTGATTTAACCGTTGGTAGCGGCTATT  
\*\* \*\*\*\*\* \*\*\* \*\*\*\*\* \*\* \*\* \*\*\*\*\* \* \*\* \* \* \*\*\*\*\* \*\* \*\* \*

BCVspike GTGTGGATTACTCTACAAAAGACGAAGTCGTAGAGCGATTACCACTGGTTATCGGTTTA  
HCVspike GTGTGGATTACTCTACAAAAGACGAAGTCGTAGAGCGATTACCACTGGTTATCGGTTTA  
CRCVspike GGGGGGATTACTCTACAAAAGACGAAGTCGTAGAACGATTACCACTGGTTATCGGTTTA  
HEVspike GTGTTGATTATGTTACAGCACTTAGATCACGTAGATCTTTTACTACAGGTTATCGGTTTA  
\* \* \*\*\*\*\* \*\*\*\*\* \* \*\* \*\*\*\*\* \* \*\*\*\*\* \*\* \*\*\*\*\* \*\*\*\*\*

BCVspike CTAATTTTGAGCCATTTACTGTTAATTCAGTAAATGATAGTTTAGAACCCTGTAGGTGGTT  
HCVspike CTAATTTTGAGCCATTTACTGTTAATTCAGTAAATGATAGTTTAGAACCCTGTAGGTGGTT  
CRCVspike CTAATTTTGAGCCATTTACTGTTAATCCAGTAAATGATAGTTTACACCCTGTAGGTGGTT  
HEVspike CTAATTTTGAACCATTTGCCGCTAATTTGGTAAATGATAGTATAGAACCCTGTTGGTGGTT  
\*\*\*\*\* \*\*\*\*\* \* \* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

BCVspike TGTATGAAATTCAAATACCTTCAGAGTTTACTATAGGTAATATGGAGGAGTTTATTCAA  
HCVspike TGTATGAAATTCAAATACCTTCAGAGTTTACTATAGGTAATATGGAGGAGTTTATTCAA  
CRCVspike TGTATGAAATTCAAATACCTTCAGAGTTTACTATAGGTAATATGGAGGAGTTTATTCAA  
HEVspike TGTATGAAATACAGATACCTTCAGAGTTTACCATTGGTAATTTAGAAGAATTCATTCAA  
\*\*\*\*\* \*\* \*\*\*\*\* \*\*\*\*\* \*\* \*\*\*\*\* \* \*\* \* \* \*\*\*\*\*

BCVspike TAAGCTCTCCTAAAGTTACTATTGATTGTTCTGCTTTTGTCTGTGGTGATTATGCAGCAT  
HCVspike CAAGCTCTCCTAAAGTTACTATTGATTGTTCTGCTTTTGTCTGTGGTGATTATGCAGCAT  
CRCVspike CAAGATCTCCTAAAGTTACTATTGATTGTTCTGCTTTTGTCTGTGGTGATTATGCAGCAT  
HEVspike CGAGTTCCCTAAGTTACTATAGATTGTGCTACATTTGTTGTGGTGACTATGCTGCAT  
\*\* \*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\* \*\*\*\*\*

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## FIGURE 9 (Page 9 of 12)

BCVspike AAGTGTTCCTCCACTACTCTCAGAAAATCAGATCAGTGGATACACTTTGGCTGCTACCT  
HCVspike AAGTGTTCCTCCACTGCTCTCAGTAAATCAGATCAGTGGATACACTTTGGCTGCCACCT  
CRCVspike AAGTGTTCCTCCACTGCTCTCAGAAAATCAGATCAGTGGATACACTTTGGCTGCCACCT  
HEVspike AAGTGTTCCTCCATTGTTATCTGAAAATCAGATTAGTGGTTACACTTCGGCAGCCACCG  
\*\*\*\*\* \* \* \* \* \*\*\*\*\* \*\*\*\*\* \* \* \* \*

BCVspike CTGCTAGTCTGTTTCCTCCTTGGTCAGCAGCAGCAGGCGTACCATTTTATTTAAATGTTCT  
HCVspike CTGCTAGTCTGTTTCCTCCTTGGTCAGCAGCAGCAGGCGTACCATTTTATTTAAATGTTCT  
CRCVspike TTGCTAGTCTGTTTCCTCCTTGGTCAGCAGCAGCAGGCGTACCATTTTATTTAAATGTTCT  
HEVspike CTGCTAGCCTATTTCTCCTTGGACAGCTGCAGCAGGCGTACCATTTTATTTAAATGTTCT  
\*\*\*\*\* \* \* \* \* \*\*\*\*\* \*\*\*\*\*

BCVspike AGTATCGTATTAATGGGATTGGTGTTACCATGGATGTTCTAAGTCAAAATCAAAAGCTTA  
HCVspike AGTATCGTATTAATGGGATTGGTGTTACCATGGATGTTCTAAGTCAAAATCAAAAGCTTA  
CRCVspike AGTATCGTATTAATGGTATTGGTGTTACCATGGATGTTCTAAGTCAAAATCAAAAGCTTA  
HEVspike AGTATCGTATAAATGGGCTTGGCGTCACCATGGATGTTCTAAGCCAAAACCAAAAGCTTA  
\*\*\*\*\* \* \* \* \* \*\*\*\*\* \*\*\*\*\*

BCVspike TTGCTAATGCATTTAACAATGCCCTTGATGCTATTAGGAAGGGTTTGATGCTACCAATT  
HCVspike TTGCTAATGCATTTAGCAATGCTCTTGATGCTATTAGGAAGGGTTTGATGCTACCAATT  
CRCVspike TTTCTAATGCATTTAACAATGCCCTTGATGCTATTAGGAAGGGTTTGATGCTACCAATT  
HEVspike TTGCTAGTGCATTTAACAACGCTCTTGATTCTATCCAGGAAGGGTTTCGACGCAACCAATT  
\*\* \* \* \* \*\*\*\*\* \* \* \* \* \*\*\*\*\* \*\*\*\*\* \* \* \* \*

BCVspike CTGCTTTAGTTAAAATTCAAGCTGTTGTTAATGCAAATGCTGAAGCTCTTAATAACTTAT  
HCVspike CTGCTTTAGTTAAAATTCAAGCTGTTGTTAATGCAAATGCTGAAGCTCTTAATAACTTAT  
CRCVspike CTGCTTTAGTTAAAATTCAAGCTGTTGTTAATGCAAATGCTGAAGCTCTTAATAACTTAT  
HEVspike CTGCTTTAGTTAAAATTCAAGCTGTTGTTAATGCAAATGCTGAAGCACTTAATAACTTAT  
\*\*\*\*\*

BCVspike TGCAACAACTCTCTAATAGATTGGTGCTATAAGTTCTTCTTTACAAGAAATTCTATCTA  
HCVspike TGCAACAACTCTCTAATAGATTGGTGCTATAAGTTCTTCTTTACAAGAAATTCTATCTA  
CRCVspike TGCRACAACTCTCTAATAAATTGGTGCTATAAGTGCTTCTTTACAAGAAATTCTATCTA  
HEVspike TGCAGCAACTCTCTAACAGATTGGTGCCATAAGTGCTTCTTTACAAGAAATTTATCCA  
\*\*\* \*\*\*\*\* \* \* \* \* \*\*\*\*\* \*\*\*\*\* \* \* \* \*

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## FIGURE 9 (Page 10 of 12)

```
BCVspike      GACTTGATGCTCTTGAAGCGCAAGCTCAGATAGACAGACTTATTAATGGGCGTCTTACCG
HCVspike      GACTGGATGCTCTTGAAGCGCAAGCTCAGATAGACAGACTTATTAATGGGCGTCTTACCG
CRCVspike     GACTTGATGCTCTTGAAGCGCAAGCTCAGATAGACAGACTTATCAATGGGCGTCTTACCG
HEVspike      GGCTCGATGCTCTTGAAGCTAAAGCTCAGATAGACAGACTTATTAATGGGCGTCTCACCG
* * * * *

BCVspike      CTCTTAATGCTTATGTTTCTCAACAGCTTAGTGATTCTACACTAGTAAAATTTAGTGCAG
HCVspike      CTCTTAATGCTTATGTTTCTCAACAGCTTAGTGATTCTACACTAGTAAAATTTAGTGCAG
CRCVspike     CTCTTAATGCTTATGTTTCTCAACAGCTTAGTGATTCTACACTAGTAAAATTTAGTGCAG
HEVspike      CTCTTAATGCTTATGTTTCTCAGCAGCTTAGTGATTCTACACTAGTAAAATTTAGTGCAG
* * * * *

BCVspike      CACAAGCTATGGAGAAGGTTAATGAATGTGTCAAAGCCAATCATCTAGGATAAAATTTT
HCVspike      CACAAGCTATGGAGAAGGTTAATGAATGTGTCAAAGCCAATCATCTAGGATAAAATTTT
CRCVspike     CACAAGCTATGGAGAAGGTTAATGAATGTGTCAAAGCCAATCATCTAGGATAAAATTTT
HEVspike      CACAAGCTATTGAGAAAGTTAATGAATGTGTTAAAGCCAATCATCTAGGATAAAATTTCT
* * * * *

BCVspike      GTGGTAATGGTAATCATATTATATCATTAGTGCAGAATGCTCCATATGGTTTGTATTTTA
HCVspike      GTGGTAATGGTAATCATATTATATCATTAGTGCAGAATGCTCCATATGGTTTGTATTTTA
CRCVspike     GTGGTAATGGTAATCATATTATATCATTAGTGCAGAATGCTCCATATGGTTTGTATTTTA
HEVspike      GTGGTAATGGTAATCATATTATATCATTAGTACAGAATGCTCCATATGGTTTGTATTTTA
* * * * *

BCVspike      TCCACTTTAGCTATGTCCCTACTAAGTATGTCACTGCGAAGGTTAGTCCCGGTCTGTGCA
HCVspike      TCCACTTTAGCTATGTCCCTACTAAGTATGTCACTGCGAAGGTTAGTCCCGGTCTGTGCA
CRCVspike     TCCACTTTAGCTATGTCCCTACTAAGTATGTCACTGCGAAGGTTAGTCCCGGTCTGTGCA
HEVspike      TCCATTTTAGCTATGTCCCCACCAAGTATGTTACAGCAAAGGTTAGTCCCGGTCTGTGCA
* * * * *

BCVspike      TTGCTGGTGATAGAGGTATAGCCCCTAAGAGTGGTTATTTTGTTAATGTAAATAACACTT
HCVspike      TTGCTGGTGATAGAGGTATAGCCCCTAAGAGTGGTTATTTTGTTAATGTAAATAAATACTT
CRCVspike     TYGCAGGTGATAGAGGTATAGCTCCTAAGAGTGGTTATTTTGTTAATGTAAATAACACTT
HEVspike      TTGCTGGCGATATAGGAATATCGCCTAAGAGTGGTTATTTTATTAATGTAAATAACTCTT
* * * * *
```

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## FIGURE 9 (Page 11 of 12)

BCVspike GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCTATAACTGGAAATAATGTTGTTG  
HCVspike GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCCATAACTGGAAATAATGTTGTTG  
CRCVspike GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCTATAACTGGAAATAATGTTGTTG  
HEVspike GGATGTTCACTGGTAGTGGCTATTACTACCCTGAACCTATAACCCAAAATAATGTTGTTG  
\*\*\*\*\*

BCVspike TTATGAGTACCTGTGCTGTTAATTACACTAAAGCACCGGATGTAATGCTGAACATTTCAA  
HCVspike TTATGAGTACCTGTGCTGTTAATTACTATACTAAAGCGCCGGATGTAATGCTGAACATTTCAA  
CRCVspike TTATGAGTACCTGTGCTGTTAATTACTATACTAAAGCACCGGATGTAATGCTGAACATTTCAA  
HEVspike TGATGAGTACGTGTGCTGTTAATTATACTAAAGCACCGGATCTAATGCTGAACACATCGA  
\* \*\*\*\*\*

BCVspike CACCCAACCTCCCTGATTTTAAGGAAGAGTTGGATCAATGGTTTAAAAACCAAACATCAG  
HCVspike CACCCAACCTCCATGATTTTAAGGAAGAGTTGGATCAATGGTTTAAAAACCAAACATCAG  
CRCVspike CACCCAACCTCCCTGATTTTAAGGAAGAGTTGGATCAATGGTTTAAAAACCAAACATTAA  
HEVspike CACCCAACCTTCCTGATTTCAAGGAAGAATTGTATCAATGGTTTAAAAACCAATCTTCAT  
\*\*\*\*\*

BCVspike TGGCACCAGATTTGTCACCTTGATTATATAAATGTTACATTCTTGGACCTACAAGATGAAA  
HCVspike TGGCACCAGATTTGTCACCTTGATTATATAAATGTTACATTCTTGGACCTACAAGATGAAA  
CRCVspike TGGCACCAGATTTGTCACCTTGATTATATAAATGTTACATTCTTGGACCTACAAGATGAAA  
HEVspike TGGCACCAGATTTGTCATTTGATTATATAATGTTACGTTCTTGGACCTACAAGATGAAA  
\*\*\*\*\*

BCVspike TGAATAGGTTACAGGAGGCAATAAAAGTTTAAATCAGAGCTACATCAATCTCAAGGACA  
HCVspike TGAATAGGTTACAGGAGGCAATAAAAGTTTAAATCAGAGCTACATCAATCTCAAGGACA  
CRCVspike TGAATAGGTTACAGGAGGCAATAAAAGTTTAAATCATAGCTACATCAATCTCAAGGACA  
HEVspike TGAATAGGTTACAAGAAGCTATAAAAGTTCTAAATCATAGCTACATCAATCTCAAGGACA  
\*\*\*\*\*

BCVspike TTGGTACATATGAGTATTATGTAAATGGCCTTGGTATGTATGGCTTTTAATTGGCCTTG  
HCVspike TTGGTACATATGAGTATTATGTAAATGGCCTTGGTATGTATGGCTTTTAATTGGCCTTG  
CRCVspike TTGGTACATATGAATATTATGTAAATGGCCTTGGTATGTATGGCTTTTAATTGGCCTTG  
HEVspike TTGGTACATATGAGTATTATGTGAAATGGCCTTGGTATGTATGGCTTTTAATTGGCCTTG  
\*\*\*\*\*

## FIGURE 9 (Page 12 of 12)

```
BCVspike      CTGGTGTAGCTATGCTTGTTTTACTATTCTTCATATGCTGTTGTACAGGATGTGGGACTA
HCVspike      CTGGTGTAGCTATGCTTGTTTTACTATTCTTCATATGCTGTTGTACAGGATGTGGGACTA
CRCVspike     CTGGCGTAGCTATGCTTGTTTTACTATTCTTCATATGCTGTTGTACAGGATGTGGGACTA
HEVspike      CTGGTGTAGTTATGCTTGTTTTACTATTCTTCATATGCTGCTGTACAGGATGTGGGACTA
```

\*\*\*\* \* \* \* \* \*

```
BCVspike      GTTGTTTTTAAGAAATGTGGTGGTTGTTGTGATGATTATAC-----
HCVspike      GTTGTTTTTAAGATATGTGGTGGTTGTTGTGATGATTATACTGGACACCAGG-----
CRCVspike     GTTGTTTTTAAGAAATGCGGTGGTTGTTGTGATGATTATACTGGACATCAGG-----
HEVspike      GTTGTTTTTAAGAAATGTGGCGGTTGTTTTGATGATTATACTGGACACCAGGAGTTGTAA
```

\*\*\*\*\* \* \* \* \*

```
BCVspike      -----
HCVspike      -----
CRCVspike     -----
HEVspike      TCAAAACTTCACATGACGATTAATTCGT
```

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FIGURE 10 (Page 1 of 5)

BCVspikepro	----MFLILLISLPMALAVIGDLKCTTVSINDVDTGVPVSSTDTVDVTNGLGTYYYVLDRV
HCVspikepro	----MFLILLISLPTAFVIGDLKCTTVSINDIDTGAPSISTDIVDVTNGLGTYYYVLDRV
CRCVspikepr	----MFLILLISLPMFAVIGDLKCTTVSINDVDTGAPSISTDVVDVTNGLGTYYYVLDRV
HEVspikepro	----MFFILLITLPSVFAVIGDLKCNTSSINDVDTGVPSSISSEVVDVTNGLGTFYVLDRV
CECVspikepr	MIVLVTCILLLCSYHTASSTSNDCRQVNVTLQDGNENLIRDFLFQNFKEEGTVVVG--
	: ***: . : . * . : : * . : : : * * *
BCVspikepro	YLNTTLLNGYYPTSGSTYRNMALKGTLLSTLWFKPPFLSDFINGIFAKVKNTKVIKNG
HCVspikepro	YLNTTLLNGYYPTSGSTYRNMALKGTLLSRLWFKPPFLSDFINGIFAKVKNTKVIKKG
CRCVspikepr	YLNTTLLNGYYPTSGSTYRNMALKGTLLSTLWFKPPFLSDFIDGVFAKVKNTKVIKDG
HEVspikepro	YLNTTLLNGYYPISGATFRNVALKGTLLSTLWFKPPFLSPFNDGIFAKVKNSRFSKHG
CECVspikepr	YYPTEVWYNCSRTATTAYEYFSNIHAFYFDMEAMENSTGNARGKPLLEHVHGEFVS--V
	* * : * . : : : . : : : . : : : * : *
BCVspikepro	VMYSEFPAITIGSTFVNTSYSVVVQPHHTNLDNKLGLEISVCQYTMCEYPHTICHPNL
HCVspikepro	VMYSEFPAITIGSTFVNTSYSVVVQPHHTNLDNKLGLEISVCQYTMCEYPHTICHPNL
CRCVspikepr	VVYSEFPAITIGSTFVNTSYSVVVQPHHTNLDNKLGLEISVCQYTMCDYPHTMCHPNL
HEVspikepro	VIYSEFPAITIGSTFVNTSYSIVVKPHTSFINGNLQGLQISVCQYTMCEYPQTICHPNL
CECVspikepr	IIYISYRDDVQHRPLLKHGLVCITESRNIDYN-SFTSSQWNSICTGNDRKIPFSVIPTDN
	::* . : . : : : . : : : . : : * : * : *
BCVspikepro	GNRRIELWHWDTGVSCLYKRNFYTDVN-----ADYLYFHFYQEGGTFYAYFTDTGVVT
HCVspikepro	GNRRVELWHWDTGVSCLYKRNFYTDVN-----ADYLYFHFYQEGGTFYAYFTDTGVVT
CRCVspikepr	GNKRIELWHWDTGVPCLYKRNFYTDVN-----ADYLYSHFYQEGGTFYAYFTDTGVVT
HEVspikepro	GNQRIELWHHDTDVVSCLYRRNFYTDVN-----ADYLYFHFYQEGGTFYAYFTDTGFVT
CECVspikepr	GTKIYGLEWNEDEFVTAYISGRSYNNWNINNNWFNNVTLYSRSSTATWQHSAAVYQGVSN
	*.: * * *.. : *.: : : * . ** : . * : . *
BCVspikepro	KFLFNVYLGTVLSHYVMP-----LTCNSAMTLEYWVTPLTSKQYLLAFNQDGVIF
HCVspikepro	KFLFNVYLGTVLSHYVLP-----LTCNSAMTLEYWVTPLTSKQYLLAFNQDGVIF
CRCVspikepr	KFLFHVYLGTVLSHYVMP-----LTCNSAMTLEYWVTPLTQYLLAFNQDGVIF
HEVspikepro	KFLFKLYLGTVLSHYVMP-----LTCDSALSLEYWVTPLTTRQYLLAFNQDGVLY
CECVspikepr	FTYYKLNNNTNGLKTYELCEDYEYCTGYATNIFAPTGVGYIPDGFSEFNNWFLTNSSSTFVS
	::: . * . * : . : . * : : : : * : . : *

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FIGURE 10 (Page 2 of 5)

BCVspikepro	NAVDCKSDFMSEIKCKTSLIAPSTGVYELNGYTVQPIADVYRR-IPNLPDCNIEAWLNDK
HCVspikepro	NAVDCKSDFMSEIKCKTSLIAPSTGVYELNGYTVQPIADVYRR-IPNLPDCNIEAWLNDK
CRCVspikepr	NAVDCKSDFMSEIKCKTSLIAPSTGVYELNGYTVQPIADVYRR-IPNLPDCNIEAWLNDK
HEVspikepro	HAVDCASDFMSEIMCKTSSITPPTGVYELNGYTVQPVATVYRR-IPDLPCNDIEAWLNSK
CECVspikepr	GRFVTNQPLLNVNCLWVPVPSFGVAAQEFCEGAQFSQCNGVFLNNTVDVIRFNLNFTADVQ
	. . . : : . * : . : : * . . * : . : : : : :
BCVspikepro	SVPSPLNWERKTFSNCFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK <sup>388</sup>
HCVspikepro	SVPSPLNWERKTFSNCFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK
CRCVspikepr	SVPSPLNWERKTFSNCFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK
HEVspikepro	TVSSPLNWERKTFSNCFNMGRMLMSFIQADSFGCN-----NIDASRLYGMCFSSITIDK
CECVspikepr	SGMGATVFSLNTTGGCILEISCYNDIVSESSFYSYGEIPFGVTDGPRYCYVLYNGTALKY
	: . . : . : * : : . . . . * . . * . . : : . : .
BCVspikepro	FAIPNGRKVDLQGLNLGYLQSFNYRIDTTATSCQLYYNLPAAN-VSVSRFNPSTWNRFRG <sup>407</sup>
HCVspikepro	FAIPNGRKVDLQGLNLGYLQSFNYRIDTTATSCQLYYNLPAAN-VSVSRFNPSTWNRFRG
CRCVspikepr	FAIPNGRKVDLQGMNLGYLQSFNYRIDTTATSCQLYYNLPAAN-VSISRFPNPSIWNRRFG
HEVspikepro	FAIPNSRKVDLQVGKSGYLQSFNYKIDTAVSSQLYYSLPAAN-VSVTHYNPSSWNRRYG
CECVspikepr	FGTLPPSVKEIAISKWQFYINGYNFFSTFPIDCISFNLTGDSGAFWTIAYTSYTEALV
	* . : : : * : . * : : . : : * : : : . : : .
BCVspikepro	FTEQSVFKPQPVGVFTDHDVVYAQHCFAKPTNFCPCKLDGSLCVGSGSIDAGYKNSGIG <sup>501</sup>
HCVspikepro	FTEQSVFKPQPVGVFTDHDVVYAQHCFAKPTNFCPCKLDGSLCVGNGPGIDAGYKNSGIG
CRCVspikepr	FTEQSVFKPQPVGVFTDHDVVYAQHCFAKPTNFCPCKLNGSLCVGSGFGIDAGYKNSGIG
HEVspikepro	FINQS-----FGSRGLHDAVYSQQCFNTPNTYCPCRT--SQCIGG-----AGTG
CECVspikepr	QVENTAIKKVTYCNSHINNIKCSQLTANLQNGFYPVASSEVGLVNKSVLLPSFYSHTSV
	: : : : : * : . : * : .
BCVspikepro	TCPAGTNYLTCH-----NAAQCNCCLCTPDPIITSKSTGPYKCPQTKYLVGIGEHCSGLAIKS <sup>525</sup>
HCVspikepro	TCPAGTNYLTCH-----NAAQCDCLCTPDPIITSKSTGPYKCPQTKYLVGIGEHCSGLAIKS <sup>528</sup>
CRCVspikepr	TCPAGTNYLTCH-----NAAQCDCLCTPDPIITSKSTGPYKCPQTKYLVGIGEHCSGLAIKS <sup>540</sup>
HEVspikepro	TCPVGTTVRKCFAAVTNATKCTCWCQPDSTYKGVNAWTCPOSKVSIPQPGQHCPGLGLIVE
CECVspikepr	NITIDLGMKRSYG--QPIASTLSNITLPMQDNNTDVYCIRSNQFSVYVHSTCKSSLWDN
	. . . : . . * : : : : : * . .

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FIGURE 10 (Page 3 of 5)

582 608  
 BCVspikepro DYCGGNPCTCQPOAFLGWSVDSCLQGDRCN--IFANFILHDVNSGTTCTDLQKSNTDII  
 HCVspikepro DYCGGNPCTCQPOAFLGWSVDSCLQGDRCN--IFANFILHDVNSGTTCTDLQKSNTDII  
 CRCVspikepr DYCGGNPCTCQPKAFLGWSVDSCLQGDRCN--IFANFILHGVNSGTTCTDLQKSNTDII  
 HEVspikepro DDCSGNPCTCKPOAFIGWSSETCLQNGRCN--IFANFILNDVNSGTTCTDLQOGNTNIT  
 CECVspikepr NFNQDCTDVLATAVIKTGTCPFSFDKLNYYLTFNKLCLSLNPTGANCKFDVAARTRTNE  
 : . . . . \* : . . . . \* \* : : \* : \* : \* : \* :

BCVspikepro LGVCVNYDLYGITGQGI FVEVNATYYNSWQNL LLYDSNGNLYGFRDYLTNRFTMIRSCYSG  
 HCVspikepro LGVCVNYDLYGITGQGI FVEVNAPYYNSWQNL LLYDSNGNLYGFRDYLTNRFTMIRSCYSG  
 CRCVspikepr LGVCVNYDLYGITGQGI FVEVNATYYNSWQNL LLYDSNGNLYGFRDYLTNRFTMIRSCYSG  
 HEVspikepro TDVCVNYDLYGITGQGI LIEVNATYYNSWQNL LLYDSSGNLYGFRDYLSNRTFLIRSCYSG  
 CECVspikepr QVVRSLYVIYEEGDNIVGVPSDNSGLHDL SVLHLD SCTDYN---IYGR TGVIIRQTNST  
 \* \* : \* : : : : . : . \* \* : \* . . : \* \* :

692 695  
 BCVspikepro RVSAAFHANSSEPALLFRNI KCNYVFNN TLSRQLQPINYFDSYLGCVVNADNSTSSAVQT  
 HCVspikepro RVSAAFHANSSEPALLFRNI KCSYVFNN TLSRQLQPINYFDSYLGCVVNADNSTSSVVQT  
 CRCVspikepr RVSAGFHSNSSEPALLFRNI KCNYVFNN TLSRQLQPINYFDSYLGCVVNADNSTSSSVQT  
 HEVspikepro RVS AVFHANSSEPALMFRNLKCSHVFNYTILRQIQLVNYFDSYLGCVVNAYNNTASAVST  
 CECVspikepr ILSGLHYTSLSGDLLGFKNVSDGVVYSVTPCDVSAQAVIDGAIVGAMTSINSELLGLTH  
 : \* . : : . \* \* \* : : . : \* : \* : : : \* : :

757 758 763 769 786  
 BCVspikepro CDLTVGSGYCVDYSTKRRSRRAITTG YRFTNFEPFTVNS-----VNDS  
 HCVspikepro CDLTVGSGYCVDYSTKRRSRRAITTG YRFTNFEPFTVNS-----VNDS  
 CRCVspikepr CDLTVGSGYWG DYSTQRRSRRITTG YRFTNFEPFTVNP-----VNDS  
 HEVspikepro CDLTVGSGYCVDYVTALRSRRSFTTG YRFTNFEPFAANL-----VNDS  
 CECVspikepr WTTTPNFYYSIYNTTNERTRGTAIDSNVDCEPIITYSNIGVCKNGALVFINVTHSDGD  
 \* . \* \* \* . \* : . . . : \* : :

792 818 827 828  
 BCVspikepro LEPVGGLYEIQIPSEFTIGNMEEFIQISSPKVTIDCSAFVCGDYAACKSQLVEYGSFCDN  
 HCVspikepro LEPVGGLYEIQIPSEFTIGNMEEFIQTSSPKVTIDCSAFVCGDYAACKSQLVEYGSFCDN  
 CRCVspikepr LHPVGGLYEIQIPSEFTIGNMEEFIQTRSPKVTIDCPVFVCGDYAACKSQLVEYGSFCDN  
 HEVspikepro IEPVGGLYEIQIPSEFTIGNLEEFITQTSSPKVTIDCATFVCGDYAACRQQLAEYGSFCEN  
 CECVspikepr VQPIS-TGNVTIPTNFTISVQVEYIQVYTTVPVSIDCSRYVCNGNPRCNKLLTQYVSACQT  
 : \* : . : : \* : : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* :

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## FIGURE 10 (Page 4 of 5)

887

BCVspikepro INAILTEVNELLDDTTQLQVANSMLNGVTLSTKLKDGVNFNVDIN-----FSPVL

HCVspikepro INAILTEVNELLDDTTQLQVANSMLNGVTLSTKLKDGVNFNVDIN-----FSPVL

CRCVspikepr INAILTEVNELLDDTTQLQVANSMLNGVTLSTKLKDGFNFNVDIN-----FSPVL

HEVspikepro INAILTEVNELLDDTTQLQVANSMLNGVTLSTKIKDGINFNVDIN-----FSSVL

CECVspikepr IEQALAMSASLENMEVDSMLFVSENALKLASVEAFNSTEHLDPYKEWPNIGGSWLGLK

\* : \* . \* : . : \* . . : \* \* : . :

933

BCVspikepro GCLGSDCNKVSSRSAIEDLLFSKVKLSDVG-FVEAYNNCTGGAEIRDLCVQSYNGIKVL

HCVspikepro GCLGSACNKVSSRSAIEDLLFSKVKLSDVG-FVEAYNNCTGGAEIRDLCVQSYNGIKVL

CRCVspikepr GCLGSECNKVSSRSAIEDLLFSKVKLSDVG-FVDAYNNCTGGAEIRDLCVQSYNGIKVL

HEVspikepro GCLGSECNRASTRSAIEDLLFDKVKLSDVG-FVQAYNNCTGGAEIRDLCVQSYNGIKVL

CECVspikepr DILPSHNSKRKYRSAIEDLLFDKVVTSGLGTVDEYKRCCTGGYDIADLVCAQYNGIMVL

. \* \* . : . \*\*\*\*\* . \* \* . : \* . : \* : . \*\*\*\*\* : \* \* : . \* \* \* \* \* \* \*

977

1011 1018

BCVspikepro PPLLSENQISGYTLAATSASLFPPWS-AAAGVPFYLVQYRINGIGVTMDVLSQNQKLI

HCVspikepro PPLLSENQISGYTLAATSASLFPPWS-AAAGVPFYLVQYRINGIGVTMDVLSQNQKLI

CRCVspikepr PPLLSENQISGYTLAATFASLFPPWS-AAAGVPFYLVQYRINGIGVTMDVLTQNQKLI

HEVspikepro PPLLSENQISGYTSAATAASLFPPWT-AAAGVPFYLVQYRINGIGVTMDVLSQNQKLI

CECVspikepr PGVANDDKMTMYTASLAGGIALGALGGGAVAIPEFAVAVQARLNYVALQTDVLNKNQKLI

\* : . : : : \* \* : . : . : . \* . . : \* \* \* \* : . : \* \* : . : : \*

1063

BCVspikepro NAFNNALDAIQEGFDATN-----SALVKIQAVVNANAEALNNLLQQLSNRF

HCVspikepro NAFSNALDAIQEGFDATN-----SALVKIQAVVNANAEALNNLLQQLSNRF

CRCVspikepr NAFNNALDAIQEGFDATN-----SALVKIQAVVNANAEALNNLLQQLSNKF

HEVspikepro SAFNNALDSIQEGFDATN-----SALVKIQAVVNANAEALNNLLQQLSNRF

CECVspikepr NAFNQAIGNITQAFGKVNDAIHQTSOGLATVAKALAKVQDVVNTQGOALSHLTVQLQNSF

. \* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \*

BCVspikepro GAISSSLQEILSRLDALEAQAQIDRLINGRLTALNAYVSQQLSDSTLVKFSAQAAMEKVN

HCVspikepro GAIGSSLQEILSRLDALEAQAQIDRLINGRLTALNAYVSQQLSDSTLVKFSAQAAMEKVN

CRCVspikepr GAISASLQEILSRLDALEAQAQIDRLINGRLTALNAYVSQQLSDSTLVKFSAQAAMEKVN

HEVspikepro GAISASLQEILSRLDALEAQAQIDRLINGRLTALNAYVSQQLSDSTLVKFSAQAIEKVN

CECVspikepr QAISSSISDIYNRLDELSADAQVDRITGRITALNAFVSQTLTROAEVRASRLAKDKVN

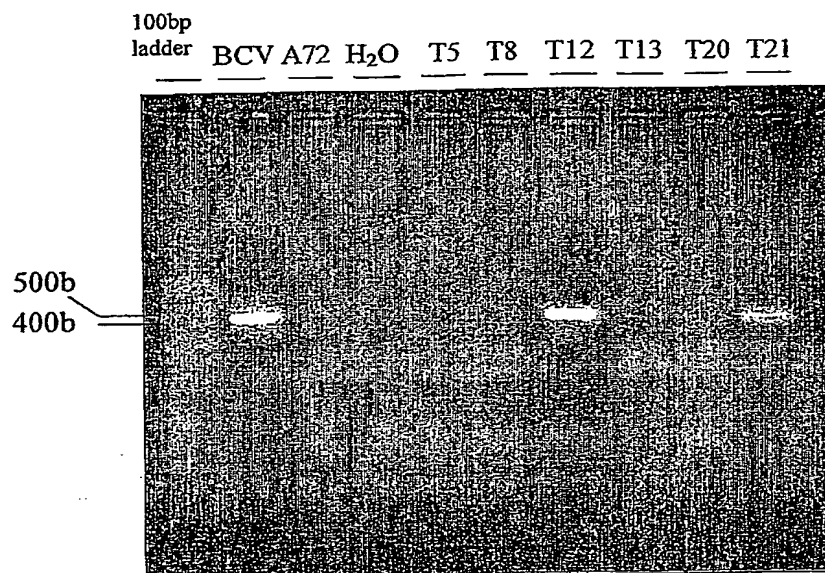
\* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \* . : \*

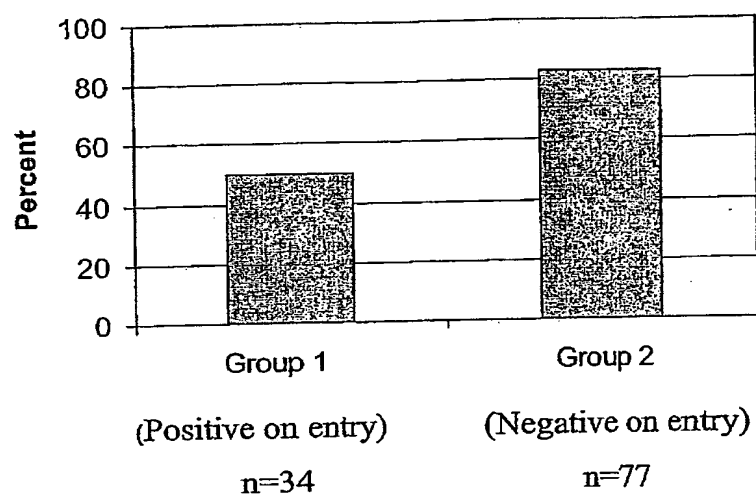


## FIGURE 10 (Page 5 of 5)

BCVspikepro	ECVKSQSSRINFCGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
HCVspikepro	ECVKSQSSRINFCGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
CRCVspikepr	ECVKSQSSRINFCGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
HEVspikepro	ECVKSQSSRINFCGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDIGIS
CECVspikepr	ECVRSQSQRFGFCGNGTHLFLSLANAAPNGMVFHTVLLPTAYETVTAWSGICASDGDRTF
	***:***.*:*****.*:***: ** *: *: * : ** * *.. .*: * :..
BCVspikepro	-----PKSGYFVNVNNTWMFTGSGYYYPEPITGNNVVMSTCAVNYTKAPDVMLNISTP
HCVspikepro	-----PKSGYFVNVNNTWMFTGSGYYYPEPITGNNVVMSTCAVNYTKAPDVMLNISTP
CRCVspikepr	-----PKSGYFVNVNNTWMFTGSGYYYPEPITGNNVVMSTCAVNYTKAPDVMLNISTP
HEVspikepro	-----PKSGYFINVNSWMFTGSGYYYPEPITQNNVVMSTCAVNYTKAPDLMLNTSTP
CECVspikepr	GLVVKDVQLTLFRNLDDKFYLTPTMYQPRAATSSDFVQIEGCDVLFVNATVIDLPSIIP
	: * *::: : * * *.. * ..* :. * * :.: * : * *
	1256 1257
BCVspikepro	NLPDFKEELDQWFKNQT--VAPDLSLDYINVTFLDLQDEM-----RLQE
HCVspikepro	NLHDFKEELDQWFKNQT--VAPDLSLDYINVTFLDLQDEM-----RLQE
CRCVspikepr	NLPDFKEELDQWFKNQT--MAPDLSLDYINVTFLDLQDEM-----RLQE
HEVspikepro	NLPDFKEELYQWFKNQSS--LAPDLSFDYINVTFLDLQDEM-----RLQE
CECVspikepr	DYIDINQTVQDILENYRPNWTVPELTIDIFNATYLNLTGEIDDLFRSEKLBNNTVELAI
	: *::: : : : * *::: * :.*::: * :.*::: * :.*::: *
BCVspikepro	AIKVLNQSYINLKDITGYEYYVKWPWYVWLLIGLAGVAMLVLLFFICCTGCGTSCFKKC
HCVspikepro	AIKVLNQSYINLKDITGYEYYVKWPWYVWLLIGFAGVAMLVLLFFICCTGCGTSCFKIC
CRCVspikepr	AIKVLNHSYINLKDITGYEYYVKWPWYVWLLIGLAGVAMLVLLFFICCTGCGTSCFKKC
HEVspikepro	AIKVLNHSYINLKDITGYEYYVKWPWYVWLLICLAGVAMLVLLFFICCTGCGTSCFKKC
CECVspikepr	LIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVFCIPLLLFCCCSTGCCG-CIGCL
	*. *: : : : * ***** :. * : : * * *.* * :
BCVspikepro	GGCCDDYTGHQELVIKTSHDD-----
HCVspikepro	GGCCDDYTGHQELVIKTSHDD-----
CRCVspikepr	GGCCDDYTGHQELVIKTSHDD-----
HEVspikepro	GGCFDDYTGHQEFVIKTSHDD-----
CECVspikepr	GSCCHSICSRQFENYEPIEKVHVH-----
	*. * .. : : : : . : .

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**FIGURE 11**

**FIGURE 12**

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## FIGURE 13

TATCGCAGCC	TTACTTTTGT	TAATGTACCA	TATGTTTATA	ATGGCTCTGC	ACAATCTACA	60
GCTCTTTGTA	AATCTGGTAG	TTTAGTTCTT	AATAACCCTG	CATATATAGC	TCGTGAAGCT	120
AATTTTGGGG	ATTATTATTA	TAAGGTTGAA	GCTGATTTCT	ATTTGTCAGG	TTGTGACGAG	180
TATATCGTAC	CACTTTGTAT	TTTAAACGGC	AAGTTTTTGT	CGAATACAAA	GTATTATGAT	240
GATAGTCAAT	ATTATTTTAA	TAAAGACACT	GGTGTTATTT	ATGGTTTCAA	TTCTACTGAA	300
ACCATTAAACA	CTGGTTTTGA	TTTTAATTGT	CATTATTTAC	TTTTACCCTC	TGTAATTAT	360
TTAGCCATTT	CAAATGAGCT	ATTGTTAAC	GTTCTACGA	AAGCAATCTG	TCTTAATAAG	420
CGTAAGGATT	TTACGCCTGT	ACAGGTTGTT	GACTCGCGGT	GGAACAATGC	CAGGCAGTCT	480
GATAACATGA	CGGCGG					497

## FIGURE 14

YRSLTFVNVP	YVYNGSAQST	ALCKSGSLVL	NNPAYIAREA	NFGDYKVE	ADFYLSGCDE	60
YIVPLCIFNG	KFLSNTKYD	DSQYFNKDT	GVIYGENSTE	TINTGFDFNC	HYLLPSGNY	120
LAISNELLLT	VPKRAICLNK	RKDFTPVQVV	DSRWNNARQS	DNMTA		165

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## FIGURE 15 (Page 1 of 2)

CRCV TATCGCAGCCTTACTTTTGTTAATGTACCATATGTTTATAATGGCTCTGCACAATCTACA  
BCV TATCGCAGCCTTACTTTTGTTAATGTACCATATGTTTATAATGGCTCTGCACAATCTACA  
OC43 TATCGCAGCCTTACTTTTGTTAATGTACCATATGTTTATAATGGCTCTGCACAATCTACA  
HECV TATCGCAGCCTTACTTTTGTTAATGTACCATATGTTTACAATGGCTCTGCACAATCTACA  
HEV TATCGCAGTCTTACTTTAGTTAATGTGCCATACGTTTACAATGGGTGAGCTCAACCCACC  
\*\*\*\*\*

CRCV GCTCTTTGTAAATCTGGTAGTTTGTCTTAATAACCCCTGCATATATAGCTCGTGAAGCT  
BCV GCTCTTTGTAAATCTGGTAGTTTGTCTTAATAACCCCTGCATATATAGCTCGTGAAGCT  
OC43 GCTCTTTGTAAATCTGGTAGTTTGTCTTAATAACCCCTGCATATATAGCTCCTCAAGCT  
HECV GCTCTTTGTAAATCTGGTAGTTTGTCTTAATAACCCCTGCATATATAGCTCGTGAAGCT  
HEV GCACCTTTGTAAAGTCTGGCAGTTTAAATCTTAACAATCCTGCATATATAGCCCGTGAGGCT  
\*\* \*\*\*\*\*

CRCV AATTTTGGGGATTATTATTATAAGGTTGAAGCTGATTTCTATTTGTCAGGTTGTGACGAG  
BCV AATTTTGGGGATTATTATTATAAGGTTGAAGCTGATTTTATTTGTCAGGTTGTGACGAG  
OC43 AACTCTGGGGATTATTATTATAAGGTTGAAGCTGATTTTATTTGTCAGGTTGTGACGAG  
HECV AATTTTGGGGATTATTATTATAAGGTTGAAGCTGATTTTATTTGTCAGGTTGTGACGAG  
HEV AATGTGGGTGATTATTATTATAAGTCTGAAGCAGATTTTCTCTCTCAGGTTGTGACGAG  
\*\* \*\* \*\*\*\*\*

CRCV TATATCGTACCACCTTTGTATTTTAAACGGCAAGTTTTGTGCGAATACAAAGTATTATGAT  
BCV TATATCGTACCACCTTTGTATTTTAAACGGCAAGTTTTGTGCGAATACAAAGTATTATGAT  
OC43 TATATCGTACCACCTTTGTATTTTAAACGGCAAGTTTTGTGCGAATACAAAGTATTATGAT  
HECV TATATCGTACCACCTTTGTATTTTAAACGGCAAGTTTTGTGCGAATACAAAGTATTATGAT  
HEV TATATCGTACCACCTTTGTATTTTAAATGGCAAGTTTTGTGCGAATACAAAGTATTATGAT  
\*\*\*\*\*

CRCV GATAGTCAATATTATTTTAAATAAAGACACTGGTGTTATTTATGGTTCTCAATTCTACTGAA  
BCV GATAGTCAATATTATTTTAAATAAAGACACTGGTGTTATTTATGGTCTCAATTCTACTGAA  
OC43 GATAGTCAATATTATTTTAAATAAAGACACTGGTGTTATTTATGGTCTCAATTCTACAGAA  
HECV GATAGTCAATATTATTTTAAATAAAGACACTGGTGTTATTTATGGTCTCAATTCTACTGAA  
HEV GATAGTCAATATTATTTTAAATAAAGACACTGGTGTTATTTATGGTCTCAATTCTACTGAA  
\*\*\*\*\*

CRCV ACCATTAACTGGTTTGTATTTTAAATTGTCATTATTTACTTTTACCCTCTGGTAATTAT  
BCV ACCATTAACTGGTTTGTATTTTAAATTGTCATTATTTAGTTTACCCTCTGGTAATTAT  
OC43 ACCATTAACTGGTTTGTATTTTAAATTGTCATTATTTAGTTTACCCTCTGGTAATTAT  
HECV ACCATTAACTGGTTTGTATTTTAAATTGTCATTATTTAGTTTACCCTCTGGTAATTAT  
HEV ACCATTAACTGGTTTGTATTTTAAATTGTCATTATTTAGTTTACCCTCTGGTAATTAT  
\*\*\*\*\*

CRCV TTAGCCATTTCAAATGAGCTATTGTTAACTGTTCCCTACGAAAGCAATCTGTCTTAATAAG  
BCV TTAGCCATTTCAAATGAGCTATTGTTAACTGTTCCCTACGAAAGCAATCTGTCTTAATAAG  
OC43 TTAGCCATTTCAAATGAGCTATTGTTAACTGTTCCCTACGAAAGCAATCTGTCTTAATAAG  
HECV TTAGCCATTTCAAATGAGCTATTGTTAACTGTTCCCTACTAAAGCAATCTGTCTTAATAAG  
HEV CTAGCCATTTCAAATGAGCTATTGTTAACTGTTCCCTACTAAAGCAATCTGTCTTAATAAG  
\*\*\*\*\*

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```

CRCV  CGTAAGGATTTTACGCCTGTACAGGTTGTTGACTCGCGGTGGAACAATGCCAGGCAGTCT
BCV    CGTAAGGATTTTACGCCTGTACAGGTTGTTGACTCTCGGTGGAACAATGCCAGGCAGTCT
OC43   CGTAAGGATTTTACGCCTGTACAGGTTGTTGATTTCGCGGTGGAACAATGCCAGGCAGTCT
HECV   CGTAAGGATTTTACGCCTGTACAGGTTGTTGACTCGCGGTGGAACAATGCCAGGCAGTCT
HEV    CGTAAGGTTTTTACGCCTGTACAGGTTGTTGATTTCGCGGTGGAACAATGCCAGGCAATCT
*****

```

```

CRCV  GATAACATGACGGCGGT
BCV    GATAACATGACGGCGGT
OC43   GATAACATGACGGCGGT
HECV   GATAACATGACGGCAGT
HEV    GATAACATGACGGCAGT
*****

```

## FIGURE 16

```

CRCV  YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
BCV    YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
OC43   YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAPQANS GDYYYKVEADFYLSGCDE
HECV   YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
HEV    YRSLTLVNVPYVYNGSAQPTALCKSGSLILNNPAYIAREANVG DYYYKSEADFSLSGCDE
*****

```

```

CRCV  YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGFNSTETINTGFDNFCHYLILPSGNY
BCV    YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTTGFDNFCHYLVLP SGNY
OC43   YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTTGFDNLNCCYLVLP SGNY
HECV   YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTTGFDNFCHYLVLP SGNY
HEV    YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTTGFDNFCHYLVLP SGNY
*****

```

```

CRCV  LAISNELLLLTVPTKAICLNKRKDFTPVQVDSRWNNARQSDNMTA
BCV    LAISNELLLLTVPTKAICLNKRKDFTPVQVDSRWNNARQSDNMTA
OC43   LAISNELLLLTVPTKAICLNKRKDFTPVQVDSRWNNARQSDNMTA
HECV   LAISNELLLLTVPTKAICLNKRKDFTPVQVDSRWNNARQSDNMTA
HEV    LAISNELLLLTVPTKAICLNKRKVFTPVQVDSRWNNARQSDNMTA
*****

```

FIGURE 17

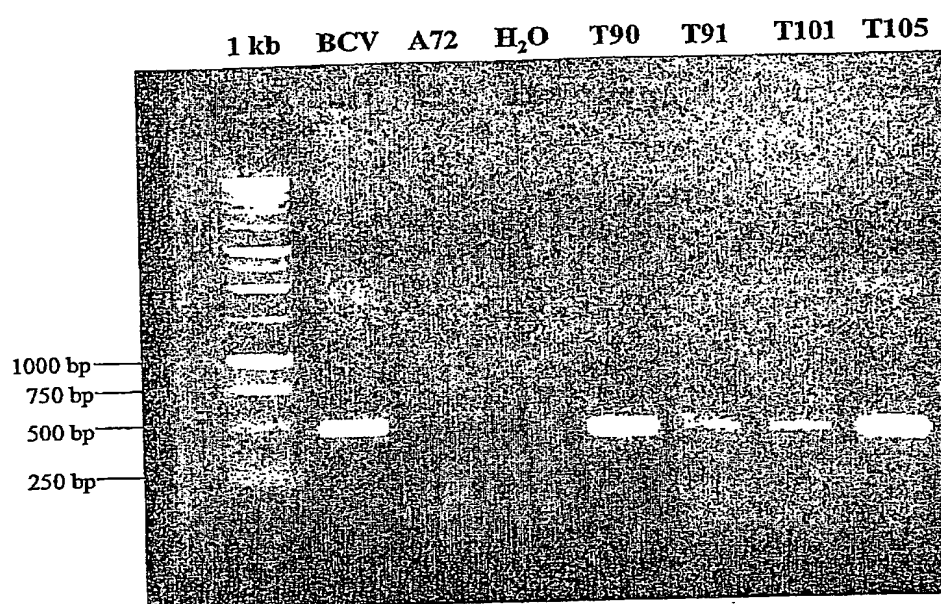
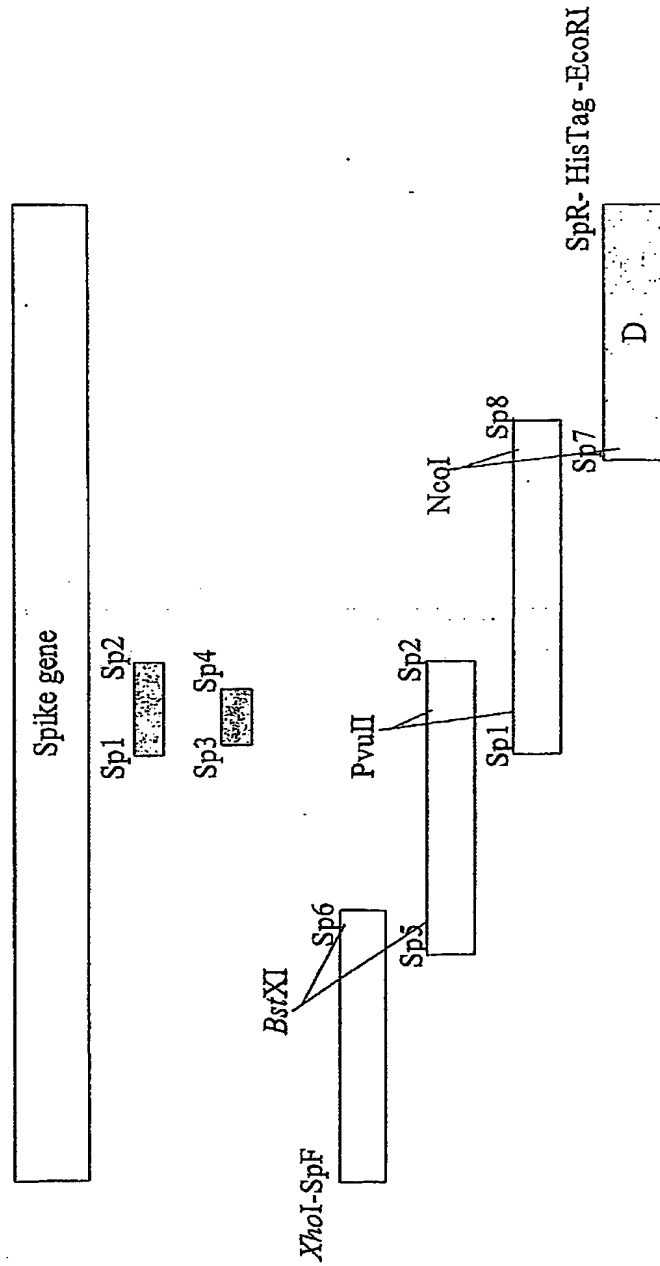


FIGURE 18





# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/EP 03/02832

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/50 C07K14/165 G01N33/50 C12Q1/68 C12N7/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STEPHENSEN, C.B., CASEBOLT, D.B., GANGOPADHYAY, N.N.: "Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay" VIRUS RESEARCH, AMSTERDAM, NL, vol. 60, no. 2, April 1999 (1999-04), pages 181-189, XP002242737 ISSN: 0168-1702 cited in the application the whole document ----- -/-	1-10, 24, 30, 31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*G\* document member of the same patent family

Date of the actual completion of the international search

20 October 2003

Date of mailing of the international search report

27/10/2003

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# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/GB 03/02832

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	"STUDIES OF RESPIRATORY DISEASE IN RANDOM SOURCE LABORATORY DOGS VIRAL INFECTIONS IN UNCONDITIONED DOGS" BINN L N—; ALFORD J P; MARCHWICKI R H; KEEFE T J; BEATTIE R J; WALL H G, vol. 29, no. 1, 1979, pages 48-52, XP0008023128 cited in the application the whole document	
A	WO 93 23423 A (SMITHKLINE BEECHAM CORP ; JONES ELAINE V (US); KLEPFER SHARON (US); MI) 25 November 1993 (1993-11-25) the whole document	1-87
X	US 5 672 350 A (BABIUK LORNE A ET AL) 30 September 1997 (1997-09-30) the whole document	47-54
A	WO 99 25838 A (CONTRERAS ANAPATRICIA ; BROWN THOMAS P (US); UNIV GEORGIA (US); VILLEG) 27 May 1999 (1999-05-27) claims 8-12	1
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Intel  
nal application No.  
PCT/GB 03/02832

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

## Continuation of Box I.1

Although claims 66 to 69 and 79 to 82 are directed to a method of treatment of the animal body, the search could be carried out and based on the alleged effects of the compound/composition.

Although claims 24 to 36, claims 67 to 69 and 87 are directed to a diagnostic method comprising steps practised on the animal body, the search has been carried out and based on the alleged effects of the compound/composition.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No  
PCT/GB 03/02832

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